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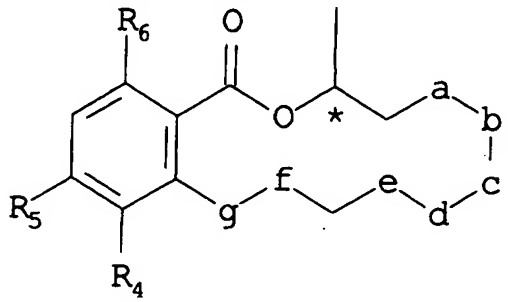
(54) Lactones compounds useful as pharmaceuticals.

(57) Novel compounds of formula I



I

wherein R_4, R_5, R_6, -a-b-, c, -d-e- and -f-g- are as defined are cytokine release inhibitors and IL-1 antagonists and are thus indicated for treatment of disorders with an aetiology associated with or comprising excessive cytokine release, particularly IL-1β release, including a wide variety of inflammatory states and diseases such as RA, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma. Related known compounds Zearalenone and radicicol and derivatives thereof have also been found to have cytokine release inhibitor properties and have similar pharmaceutical applications.

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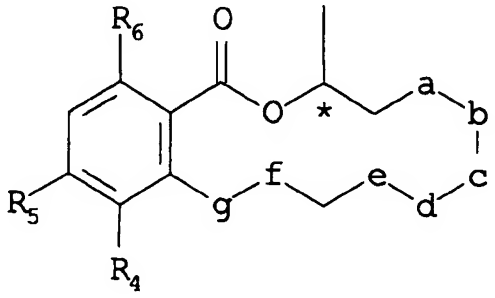
This invention relates to compounds useful as a cytokine release inhibitors and IL-1 antagonists. The invention provides the novel compounds of formula I



I

in which R_4 and R_6 are the same or different and are H, OH, C_{1-4} alkoxy or C_{1-4}alkyl COO-,

R_5 is OH, C_{1-4}alkoxy or C_{1-4}alkyl COO,

one of -a-b- or -d-e- is $-CHR_7-CHR_8-$ and the other is cis- or trans- $-CR_7=CR_8-$, wherein R_7 and R_8 are the same or different and are H, OH, C_{1-4} alkoxy or C_{1-4}alkyl COO-,

c is CH-OH or C=O and

-f-g- is $-CH_2-CH_2-$ or cis- or trans- -CH=CH-

provided that, when R_4 is H, R_6 is OH and -f-g- is trans- -CH=CH-; 1. R_5 is not OH when -a-b- is $-CH_2-CH_2-$, c is C=O and -d-e- is $-CH_2-CH_2-$, or 2. R_5 is not methoxy when -a-b- is $-CH_2-CH_2-$ or cis- -CH=CH- and c is C=O or CH-OH and -d-e- is -CHOH-CHOH,

in free form or base salt form or in the form of a physiologically-hydrolysable and -acceptable ester and wherein the asymmetric carbon marked * and the atoms a and/or b or d and/or e, when these are asymmetric carbon atoms have the R- or S-configuration or the compound comprises any mixture of the optical isomers thereof.

Preferably R_4 and R_6 are the same or different and are H, -OH, MeO- or Me-COO-. Preferably R5 is -OH, MeO- or MeCOO-. More preferably R_4 is H or MeO; R_5 is MeO, and R_6 is OH or MeO.

Preferably -a-b- is cis- or trans- $-CR_7'=CR_8'-$, wherein R_7' and R_8' are the same or different and are H, OH, MeO- or Me-COO-. More preferably -a-b- is cis- or especially trans- -CH=CH-.

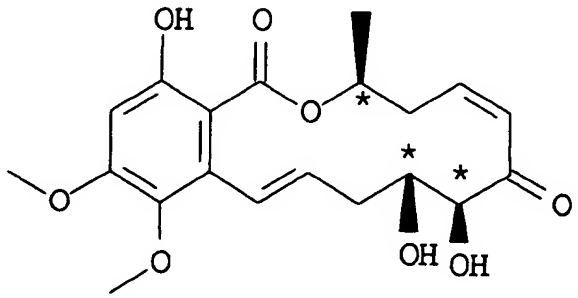
Preferably -d-e- is $-CHR_7'-CHR_8'-$, wherein R_7' and R_8' are as defined above. More preferably -d-e- is $-CH_2-CH_2-$ or especially -CHOH-CHOH-, wherein the OH groups may be in free or protected form.

Most preferably -f-g- is trans- -CH=CH-.

Preferably the asymmetric carbon atoms of the compounds of the invention all have the S-configuration.

In particular embodiments the invention provides compounds of formula I in which R_4 is H or methoxy, R_5 is methoxy, R_6 is OH, -a-b- is cis- or trans- -CH=CH-, c is CHOH or C=O, -d-e- is -CHOH-CHOH- and -f-g- is trans- -CH=CH-; provided that when -a-b- is cis -CH=CH-, then R_4 is methoxy and c is C=O in free form or base salt form or in the form of a physiologically-hydrolysable and -acceptable ester.

Formula I', which is formula I in which R_4 and R_5 are methoxy, R_6 is OH, -a-b- is cis- -CH=CH-, c is C=O, -d-e- is -CHOH-CHOH-, -f-g- is trans- -CH=CH- and the asymmetric carbon atoms marked * all have the S-configuration

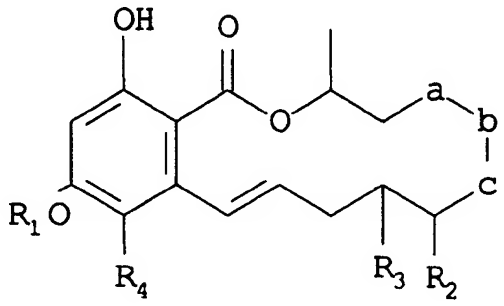


I'

is the structure which has been assigned to the novel metabolite designated 87-250904-F1 and having the following characteristics:

White needles (from methanol/water 1:1), m.p. 173-174°C,
$[\alpha]_D^{25}$ = -43.6°(MeOH, c = 0.76)
Mass spectrum (FAB) : m/e = 393 (MH^+)
IR spectrum in KBr : see Fig. 1
Proton NMR in $CDCl_3$, 360 MHz with TMS as internal standard, see Fig. 2
Solubility: Almost insoluble in water, readily soluble in methanol, DMSO, chloroform.
HPLC : Column : LiChrospher 100 RP-8 (5µm) (LiChroCART 125-4, Merck)
Mobile phase: acetonitrile/water/orthophosphoric acid 350:650:0.175 (by vol.)
Flow rate: 1.0 ml/min
Detection: UV 210 nm
Retention time: 3.8 min.

The compounds of formula I are novel compounds which belong to the same class of compounds as zearalenone, which is the compound of formula II

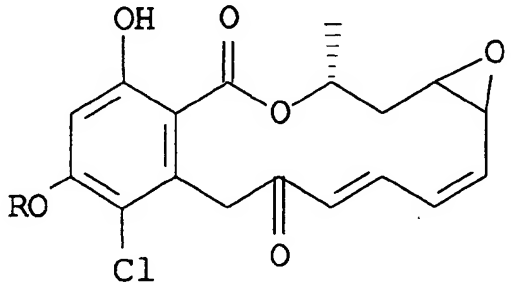


II

in which $R_1 = R_2 = R_3 = R_4$ = H, -a-b- = $-CH_2-CH_2-$, and c = C=O. Zearalenone is known to have anabolic effects. The compounds of formula II in which R_1 is methyl, $R_2 = R_3$ = OH, R_4 = H, -a-b- = $-CH_2-CH_2-$ or cis- -CH=CH- and c = C=O or CH-OH are described by Ellestad et al, J. Org. Chem. **43** 2339 (1978), where they are stated to have "no particularly interesting activities" and it is speculated that the presence of the phenolic ether (i.e. the methoxy group on the benzene ring) may be responsible for this lack of activity.

In view of this it is surprizing that the compounds of formula I should have any pharmacological activity.

The compounds of formula I are also related to the compound radicicol, which is the compound of formula III



III

in which R is H. The corresponding methoxy compound in which R is methyl is also known.

Radicicol, a metabolite of Monosporium bonorden, is known to have antibiotic properties [Delmotte, Nature **171** 344 (1953)]

Surprizingly, it has now been found that the compounds described above, namely the novel compounds of Formula I, radicicol, O-methyl radicicol and the zearalenone derivatives described by Ellestad et al also have cytokine release inhibitor properties, in particular, they inhibit the release of IL-1, IL-6 and TNF-α, and also act as functional antagonists of IL-1.

The compound of formula I' may be produced by cultivating a producing microbial strain in a nutrient medium. Preferred micro-organisms are strains of pycnidial imperfect fungi, in particular the strain F/87-250904, which produces the metabolite 87-250904-F1.

This strain has been isolated from an unidentified lichen collected in South Africa, and a viable culture of the strain was deposited on 6 Nov. 1991 at the ARS Patent Culture Collection, US Dept. of Agriculture, Northern

Regional Research Center, Peoria, Illinois, USA under the provisions of the Budapest Treaty and was given the reference number NRRL 18919. The culture may also be obtained from Sandoz Ltd., Basle, Switzerland.

The fungal strain NRRL 18919 grows on most usual fungal agar media such as 2 % malt extract agar. The temperature range for growth is between approx. 5 and 37°C, the optimal temperature for growth is between approx. 24 and 32°C. On 2% malt extract agar in petri dishes and at 27°C strain NRRL 18919 will form after 10 days incubation colonies 25 to 35 mm in diameter. The colonies appear brown to greenish black to black with moderately developed aerial mycelium. No pronounced diffusable pigments are formed under these conditions. Strain NRRL 18919 can degrade starch and keratin, but not or only to a very limited degree cellulose and chitin.

The pycnidia of strain NRRL 18919 are very variable in shape and size. They range from approx. 30µ large, globose to pyriform with a single ostiolum to irregularly shaped, up to 600µ large pycnidia or pycnidial complexes with several ostioli. The outer pycnidial walls are composed of brown cells. The conidia are hyaline, aseptate, bacilliform, reniform to allantoid, often constricted and 3.0 - 5.5 x 1.0 - 1.7µ large. Conidia are produced within the pycnidia by slender phialides and collect outside the ostioli in a whitish mass. The strain NRRL 18919 can best be accomodated in the genus Phoma Sacc. and fits quite well to the description of Phoma cava Schulzer.

The new strain NRRL 18919 may be cultured at suitable temperatures in various culture media using appropriate nutrients and mineral substances, as aerobic surface or immersion cultures. The invention also provides fermentation broths which are obtained by cultivation of an 87-250904-F1 producing fungal strain, particularly of the strain NRRL 18919. A further aspect of the invention provides a process for the preparation of the compound of Formula I comprising the steps of cultivating an 87-250904-F1-producing fungal strain and isolating the metabolite 87-250904-F1 which is formed.

The fermentation media should contain a utilisable source of carbon and optionally mineral salts and growth factors, all of which can be added in the form of well defined products or as complex mixtures, as are found in biological products of various origins.

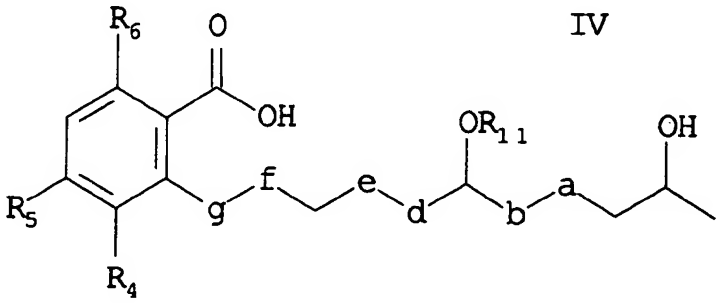
In order to produce the new metabolite 87-250904-F1, strains may also be used which are obtained by selection or mutation under the influence of ultra-violet radiation, X-rays or by other means, e.g. by the use of chemical mutagens.

As soon as a sufficient amount of metabolite has accumulated in the culture, it may be concentrated and isolated in conventional manner, for example by extraction and subsequent chromatographic methods.

Compounds of formula I in which a-b is trans- -CH=CH- may be prepared from the corresponding compounds in which a-b is cis- -CH=CH- by isomerization under mild alkaline conditions, for example in solution in pyridine, suitably for 12 - 48 hr at temperatures between 0 and 80°C, preferably at about 50°C.

The compounds of formula I may also be prepared by complete or partial chemical synthesis using conventional synthesis techniques.

Thus in a further aspect the invention provides a process for the preparation of a compound of formula I, in which c is CHOH, which comprises cyclising a compound of formula IV

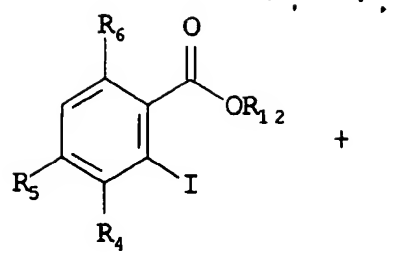
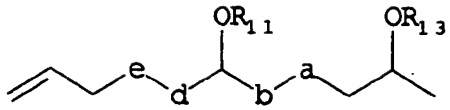


IV

wherein R_4, R_5, R_6, -a-b-, -d-e- and -f-g- are as defined above with the exception that any OH substituents on -a-b- or -d-e- are in suitably protected form, and R_{11} is H or an OH protecting group;
and removing any OH protecting groups therefrom.

Compounds of formula I in which c is C=O may be prepared from the the cyclised product by oxidation of the CHOH group at c.

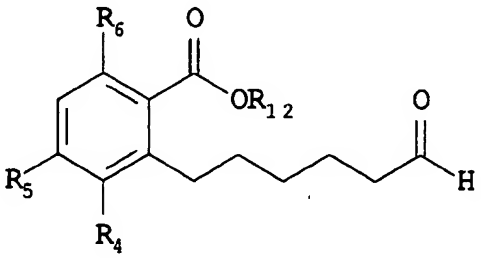
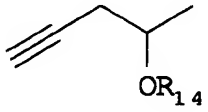
The compounds of formula IV wherein -f-g- is trans- -CH=CH- may be prepared by linking compounds of formulae V and VI

V  + VI 

wherein R_4, R_5, R_6, R_{11}, -a-b-, -d-e- and -f-g- are as defined above for formula IV and R_{12} and R_{13} are OH protecting groups,
and removing the R_{12} and R_{13} are OH protecting groups.

Compounds of formula VI may be prepared by linking an hydroxy protected analogue of 4-hydroxybut-1-yne with hex-1-en-5-one, partially or completely reducing the acetylene bond and, if appropriate, adding an R_{11} OH protecting group.

The compounds of formula IV wherein -f-g- is $-CH_2-CH_2-$ may be prepared by linking compounds of formulae VII and VIII

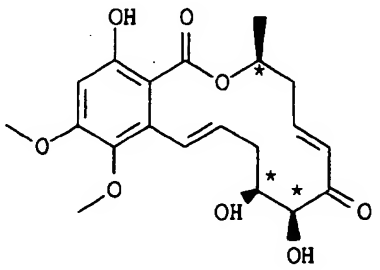
VII  + VIII 

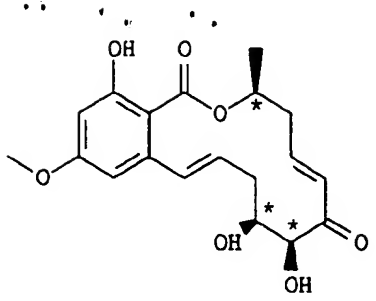
wherein R_4, R_5, R_6, and R_{12} are as defined above for formulae IV and V and R_{14} is an OH protecting group,
partially or completely reducing the acetylene bond and if appropriate removing OH protecting groups.

The compound of formula VII may be prepared by linking a compound of formula V as above with an hydroxy protected analogue of 6-hydroxy-hex-1-ene, reducing the alkylene bond corresponding to f-g in formula I, removing the protecting group from the terminal hydroxy group on the C_6 side chain and oxidising this hydroxy group to an aldehyde group.

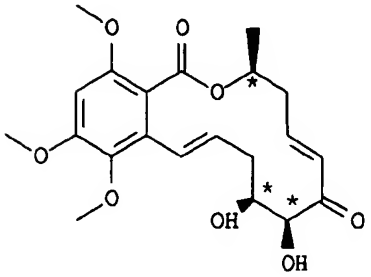
The processes described above may be carried out using conventional synthesis procedures, reagents and conditions; for instance, as described hereinafter in the Examples.

Preferred compounds in which a-b is trans- -CH=CH- are the compounds of formulae I", X and XI.

 I"

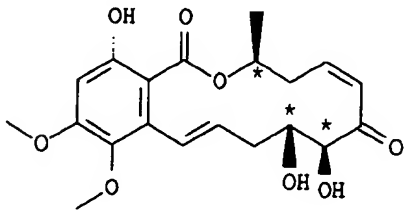


X

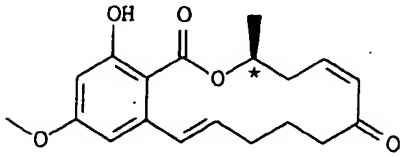


XI

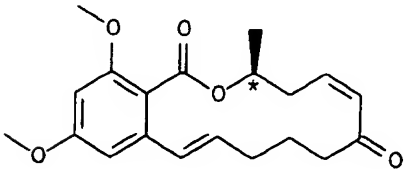
Preferred compounds in which a-b is cis- -CH=CH- are the compounds of formulae I', XII and XIII.



I'



XII



XIII

The compounds of the invention have pharmacological properties. In particular, they show cytokine inhibitory effects, acting not only to inhibit the release of IL-1, IL-6 and TNF-α, but also as functional antagonists of IL-1 as indicated in the following in vitro and in vivo test methods:

1. Cytokine Release from THP-1 Cells

The THP-1 cell line is generally available and is described by Tsuchiya et al, Int. J. Cancer 26 171-176 (1980). 900 µl THP-1 cells (0.5 x 10^6 cells) together with 100 U γ-interferon/0.9 ml RPMI 1640 medium (containing 2 mM L-glutamine and 5 % heat-inactivated foetal calf serum) are pipetted into 24 well culture plates. 100 µl of the compound to be tested are then added. After 3 hours at 37°C in 5 %

CO_2/95 % air, 10 µl lipopolysaccharide 500 µg/ml is added and the incubation continued for a further 40 hours. Appropriate controls (with and without stimulus, solvent) are also included. The media are then removed and clarified by centrifugation at 1000 g for 10 min. 1.0 ml digitonin 0.01 % is added to the wells to lyse the cells which are loosened by scraping with a rubber policeman and left at 4°C for 10 min. Lactate dehydrogenase measurements are then performed immediately and the samples stored at -20°C until the other determinations can be made. The assays are: IL-1β (medium and lysate), IL-6 (medium), TNF-α (medium), PGE2 (medium and lysate), lactate dehydrogenase (LDH) (medium and lysate) and DNA (lysates) . IL-1β, IL-6 and TNF-α assays are determined using commercially available ELISA kits (Cistron), PGE2 is measured using a standard RIA and DNA fluorimetrically using DAPI (4',6-diamidino-2-phenylindole.2HCl)

In this test, the compounds of the invention inhibit IL-1β ,Il-6, TNF-α and PGE2 release at a concentration of about 0.001 to 10 µM. In contrast DNA levels remain substantially unaffected, and the compounds are non-toxic, since LDH release is unchanged.

2. Cytokine Release from Human Monocytes

a) Human Monocytes

Mononuclear cells are obtained from the blood of healthy volunteers via centrifugation and cultivated on tissue culture dishes with the test compound at various concentrations [Schnyder et al., Agents & Actions, **30**, 350-362 (1990)]. The non-adherent lymphocytes are removed after 4 hr. by washing several times. Fresh medium, test compound and LPS (10 µg/ml) as stimulant are added and the monocytes incubated for a further day. The pooled culture media are diluted 1:10 with fresh medium and added to confluent rabbit chondrocytes. Metallo- proteinase (MP) activity in the chondrocyte culture medium is assayed after a further 2 days as described below. Compounds of the invention are active in suppressing monokine release in this test method at a concentration of the order of from 0.001 to 10µM.

b) Determination of IL-1 by the chondrocyte test

Purified IL-1, recombinant human IL-1-β (rhIL-1) or conditioned media collected from stimulated human monocytes, mouse macrophages or mouse cell line $P388D_1$, cause characteristic changes in the secretory pattern of chondrocytes. In particular, a latent metalloproteinase (MP) is induced, whilst secretion of plasminogen activator is reduced. The property of the metallo-proteinase or stromelysin has been described in detail [Chin et al., J. Biol. Chem. **260**, 12367 - 12376 (1985)], as has that of the plasminogen activator [Schnyder et al., Analyt. Biochem. **200**, 156-162, 1992]. Dose-response curves using purified or recombinant IL-1 and neutralisation with an antibody to human monocyte IL-1 have shown that this system can be used as a specific and sensitive bioassay for IL-1. Stimulation of the secretion of a latent metalloproteinase by rabbit articular chondrocytes is relatively IL-1-specific, IL-2, TNF-α, recombinant human interferon-α, and -γ, phorbol myristate acetate, Concanavalin A, E-type prostaglandin and indomethacin having no influence [Schnyder and Payne, Brit. J. Rheumatol. **24** (suppl. 1), 128 - 132 (1985); Schnyder et al., J. Immunol. **138**, 496 - 503 (1987)].

Chondrocytes are harvested and cultured as described [Evequoz et al., Biochem. J. **219**, 667 - 677 (1984)]. Briefly, chondrocytes are released from slices of distal femur articular cartilage from ca. 1.2 kg female New Zealand White rabbits by treatment with proteinases. The washed cells are cultured on 48-well culture plates in DMEM, enriched with 1 % antibiotics, 2 mM glutamine and 10 % heat-inactivated fetal calf serum. After reaching confluency the cells are incubated with 20 µl samples of the test culture media for IL-1 bioassay and made up to a volume of 300 µl Iscove's modified Dulbecco's medium. The supernatant media are collected after 48 h, centrifuged and processed for biochemical analysis.

c) Biochemical Assays

Metalloproteinase (MP) is measured kinetically by using a 96-well plate Twinreader (Flow Laboratories AG) linked to a personal computer. Ac-Pro-Leu-Gly-S-Leu-Leu-Gly-OC2H5, a synthetic substrate for vertebrate collagenase is used for the determination of MP [Weingarten and Feder, Anal. Biochem. 147, 437-440 (1985)]. 50 µl of the latent MP is activated with 50 µl trypsin (120 µg/ml in 50 mM PIPES pH 6.8, containing 20 mM CaCl2) for 30 min at 37°C, after which time the activities of all serine proteinases are stopped by adding 150 µl Soybean trypsin inhibitor (SBTI; 166 µg/ml in the above buffer). A 50 µl aliquot of the activated MP is then mixed with 100 µl reagent solution (2.5 mM 5,5'-dithio-bis-2-nitrobenzoic acid; DTNB, 100 µg/ml SBTI, 20 mM CaCl2 in the above buffer), and kept for 10 min at room temperature in order to react with all free SH-groups. The reaction is then started by adding 100 µl substrate solution (1.25 mM in buffer containing 100 µg/ml SBTI) and the changes in absorbance at 414 nm is measured 11 times at 1 min intervals.

3. Functional IL-1 Antagonistic Effects

The test method described in 2) above is repeated, but instead of adding human monocytes to the chondrocyte culture, IL-1 itself (recombinant human IL-1β, Sandoz) is added at a concentration of 1 ng/ml. MP activity in the chondrocyte culture medium is assayed after 2 days. Compounds of the invention are active as functional IL-1 antagonists at concentrations of 1-30 nM.

4. LPS-Fever

An LPS-suspension (Sigma, No. L-5886; 100 ug/5 ml glucose solution/kg s.c.) is injected in male Tuttlingen SD rats (150-160 g). Two hours later the body temperature is measured using a thermistor rectal probe connected to an ELLAB telethermometer. After 4 hr the test compound is administered. Two hours later (6 hr. after LPS administra- tion) the temperature is measured again. The temperature increment shown by the untreated controls is taken as 100% and that in the treated group is expressed as a percentage of this value. The ED50 is the dose causing a 50% inhibition of the temperature increase determined in the control rats. Compounds of the invention typically have an ED50 in this assay from about .1 to about 1 mg/kg.

5. Carrageenan-Induced Paw Edema in the Rat

Five OFA male rats, 150-170 g body weight, are used for each group. The test compound is administered orally as a suspension in physiological saline/0.5% tragacanth one hour prior to the carrageenan injection. Carrageenan (0.1 ml of a 1% suspension in physiological saline) is given by subplantar injection into one hind paw. The swelling of the paw is measured by means of an antiphlogometer according to Kemper & Amelm. A control reading is taken immediately after the injection, and the swelling is measured after 3 and 5 hrs. The mean value of the 3- and 5-hour reading is taken after deduction of the control reading, and the values obtained from the treated animals are expressed as a percentage of the value obtained from non-treated controls. The ED50 is the dose causing a 50% inhibition of the carrageenan-induced swelling after 3 hr. Compounds of the invention typically have ED50s in this assay of from about .1 to about 1 mg/kg.

The compounds of the invention are therefore indicated for the treatment of disorders with an aetiology associated with or comprising excessive cytokine release, particularly IL-1β release, e.g. in a wide variety of inflammatory states and diseases such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma.

For the above uses the required dosage will of course vary depending on the mode of administration, the particular condition to be treated and the effect desired. In general however, satisfactory results are achieved at dosage rates of from about 0.1 to 20 mg/kg animal body weight, preferably 0.5 to 5 mg/kg. It has been determined that the compound of Formula I' has an IC_{50} value in the THP-1 test (concentration of compound which inhibits to 50 % the release of IL-1β) of 0.06 μM. It is therefore indicated that this compound may be administered at daily dosages of from 8 mg to 2 g p.o. to larger mammals, for example humans.

In accordance with the foregoing the present invention also provides:

i) A method for the treatment of disorders with an aetiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma, in a subject in need thereof, which method comprises administering to said subject an effective amount of

a) a compound of Formula I as defined above; or

b) a compound of formula II in which:

R_1 is H or methyl;

R_2 and R_3 are H or OH;

R_4 is H or methoxy;

-a-b- is $-CH_2-CH_2-$ or -CH=CH-

and c is C=O or CH-OH (other than compounds of formula I) ; or

c) a compound of formula III in which R is H or methyl

in free or base salt form, or a physiologically-hydrolysable and -acceptable ester thereof.

ii) A compound of formula II in which:

R_1 is methyl; R_2 and R_3 are H or OH; R_4 is H or methoxy; -a-b- is $-CH_2-CH_2-$ or cis- -CH=CH-; and c is C=O or CH-OH; in free base or salt form, or a physiologically-hydrolysable and -acceptable ester thereof for use as a pharmaceutical, for example for use in treating any of the diseases disclosed above.

The compounds of the invention may be administered by any conventional route, in particular nasally, enterally, preferably orally, e.g. in the form of tablets or capsules, or parenterally e.g. in the form of injectable

solutions or suspensions or in a suppository form. Unit dosage forms contain, for example from about 2 mg to 1 g of the compound of the invention. The invention also provides a pharmaceutical composition comprising an effective amount of the compound of formula II as defined in b) above, in association with a pharmaceutically acceptable diluent or carrier. Such compositions may be manufactured in conventional manner.

The preferred compounds for pharmaceutical use are the compounds of formulae I', I", X, XI, XII and XIII.

The following examples illustrate the invention:

Examples

Example 1: Fermentation example

A seed culture of strain NRRL 18919 is inoculated into a 2 l Erlenmeyer flask containing 1 l of sterile, liquid medium consisting of 2% malt extract and 0.4% yeast extract in de-ionized water. This Erlenmeyer flask is incubated on a rotary shaker at 200 rpm at 24°C for three days and serves as the preculture for a 50 liter fermentation. This intermediate culture is carried out in a 75 l fermenter containing 50 l of a sterile liquid medium consisting of 2% malt extract and 0.4% yeast extract in de-ionized water. During the fermentation the temperature is held constant at 25°C, the stirrer speed at 50 rpm, the aeration at 50 l/min and the overpressure at 0.5 bar. The preculture fermentation lasts for only one day after which it is used to inoculate a final fermentation at larger scale. This production culture is carried out in a 5000 l fermenter containing 3000 l of a sterile liquid medium composed of (g/l) 20 glucose, 2 soy-protein, 2 malt extract 2 yeast extract, 2 KH_2PO_4 and 2 $MgSO_4$ in de-ionized water. Prior to inoculation 1 mg/l Cyclosporin A dissolved in a small volume of methanol is added to the sterile medium. During the fermentation the temperature is held constant at 25°C, the stirrer speed at 25 rpm, the aeration at 3000 l/min and the overpressure at 0.5 bar. Exessive foaming is prevented by controlled addition of antifoam agent. The production of the compound of the invention is monitored by HPLC and the production culture is harvested at the time of highest titers of the desired metabolite.

Example 2: Isolation of metabolite

The mycelium is separated from the culture broth and the supernatant (2800 1) is extracted with 2800 l of ethyl acetate. The mycelium is discarded. The ethyl acetate solution is evaporated to dryness under vacuum at 45-50°C. The compound of interest can be gained directly by crystalisation out of the extraction residue, by dissolving the extraction residue (ca. 1 kg) in 5 l of methanol at 60°C, cooling the resultant solution down suddenly and afterwards keeping it at room temperature for 20 hours. The resulting crystals (ca. 250 g) are separated by filtration, redissolved in methanol at 75°C and re-crystalised overnight at -25°C. The crystals are suction-filtred, washed with cold methanol and dried under vacuum. The product is [3S-(3R*, 5Z, 8R*, 9R*, 11E)]-3,4,9,10-tetrahydro-8,9,16-trihydroxy-13,14-dimethoxy-3-methyl-1H[2] benzoxacyclotetradecin-1,7[8H] dione, and is obtained in the form white needles having the following properties: m.p. 170-172°C, $[\alpha]_D^{20}$ = -43.1° (c = 0.49 in methanol).

Example 3: Preparation of the compound of formula I"

The compound of formula I", [3S-(3R*, 5E, 8R*, 9R*, 11E)]-3,4,9,10-tetrahydro-8,9,16-trihydroxy-13,14-dimethoxy-3-methyl-1H-2-benzoxycyclotetra-decin-1,7 (8H)-dione, is prepared as follows.

The product of Example 2 (1 g) is dissolved in 100 ml pyridine and stirred under an argon atmosphere for 24 hr. at 50°C. The resulting yellow solution is concentrated under vacuum and the residue is taken up in ethyl acetate and shaken with 1N tartaric acid. The organic phase is separated, washed with brine, dried over sodium sulphate and evaporated. The solid residue is chromatographed on Kieselgel using ethyl acetate as mobile phase to give the compound of formula I", m.p. 171-173°C. NMR ($CDCl_3$): 1.47 (d, 3H), 2.25-2.85 (m, 4H), 3.60 (s,3H), 3.89 (s,3H), 4.17 (m,1H), 4.65 (d,1H), 5.40 (m,1H), 5.93 (m,1H), 6.45 (s,1H), 6.47 (d,1H) 6.63 (dd,1H), 7.00 (dt,1H), 11.21 (s,1H) ppm.

Example 4: Preparation of the compound of formula X

By analogy with Example 3, the compound of formula X, [3S-(3R*, 5E, 8R*, 9R*, 11E)]-3,4,9,10-tetrahydro-8,9,16 trihydroxy-14-methoxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione, is obtained.
M.p. 154-159°C.

Example 5: Preparation of the compound of formula XI

By analogy with Example 3 the compound of formula XI, [3S-(3R*,5E,8R*,9R*,11E)]-3,4,9,10-tetrahydro-8,9-dihydroxy-13,14,16-trimethoxy-3-methyl-1H-2-benzoxacyclotetradecin-1,7(8H)-dione, is obtained as a foam having the following NMR spectrum. NMR ($CDCl_3$):1.4(d,3H); 2.2-3.0(m, 5H); 3.61(s,3H); 3.78(s,3H); 3.83(s,3H); 3.7-4.15 (m,2H); 4.75(d,3H); 2.2-3.0 (m,lH); 6.15(d,lH); 6.31(d,lH); 6.38(s,1H); 7.85(m,1H)ppm.

Example 6: Preparation of the compound of formula XIII

a). Pent-1-en-5-one

10 g (100 mMol) of 5-hydroxypent-1-ene in 100 ml of dichloromethane is added to a suspension of 43 g of pyridinium chlorochromate in dichloromethane at room temperature. After 1 1/2 hr diethylether is added, the suspension is filtered over silica gel, the solid residue washed several times with diethylether which is also filtered over silica gel, and the diethylether fractions are combined and evaporated slowly to yield 7. g of crude pent-1-en-5-one.

b). 4-Trimethylsiloxyhex-1-yne

A mixture of 8.1 g of 4-hydroxyhex-1-yne, 16 g of t.butyldimethylsilyl chloride and 7.2 g of imidazole in 100 ml of acetonitrile is stirred overnight at room temperature, after which diethylether is added and the suspension filtered over silica gel. The filtrate solution is then evaporated and the residue distilled under water pump vacuum at 70°C to give 17.8 g (93%) of the title product.

c). 2-Trimethylsiloxy-7-hydroxy-undeca-4-yn-10-ene

A solution (37 ml) of butyl lithium is added dropwise at -78°C to a solution of 13.8 g of the product of part (b) above in 20 ml of tetrahydrofuran and the mixture is stirred at -78°C for 1 1/2 hr. A solution of 6.0 g of the aldehyde product of part a) above in 10 ml of tetrahydrofuran is then added slowly, the mixture stirred at -78°C for 1 hr and then allowed to warm to room temperature. Water is then added, the mixture extracted with ethyl acetate and the organic phase dried, filtered and evaporated to give 13.1 g (72%) of the title product in impure form.

d). 2-Trimethylsiloxy-7-hydroxy-undeca-cis-4,10-diene

To a solution of 10.1 g of the product of part c) above in 300 ml of pyridine is added 1 g of 10% palladium in $BaSO_4$ and the mixture is hydrogenated at room temperature. After 24 hr 420 ml of hydrogen is taken up and after a further 24 hr 720 ml of hydrogen is taken up. The mixture is then suction filtered over Hyflo to remove the catalyst, the product concentrated, dissolved in ethyl acetate, washed with 10% citric acid solution and saturated brine, dried over Na_2SO_4 and concentrated. At this point, however, thin layer chromatography indicates that only 15% of the starting material has been converted. The product is, therefore redissolved in 300 ml of pyridine and 1.5 of 10% palladium in $BaSO_4$ is added and the mixture hydrogenated with stirring at room temperature. It is now found that already after 6 hr 700 ml of hydrogen has been taken up. The product (10.2 g) is found to be about 90% converted as judged by thin layer chromatography.

e). 2-Trimethylsiloxy-7-{(2-methoxy)ethoxy}methoxy-undeca-cis-4,10-diene

10 g of the title product of step d) above is dissolved in 80 ml of dichloromethane with stirring at room temperature and 8.5 ml of diisopropylethylamine is added dropwise over 1 min followed by 5.7 ml of 1-chloromethyl-2-methyl-glycol in 20 ml of dichloromethane dropwise over 10 min. After 2 hr the reaction is complete as determined by thin layer chromatography. The reaction mixture is washed with 10% $NaHCO_3$, 10% citric acid solution and saturated brine, dried over Na_2SO_4 and concentrated to 14 g of the title product in rude form. The product is purified by silica gel chromatography (hexane/diethylether 8:1)with a yield of 10.5 g (83%).

f). The compound of formula IV in which R_4 is H, R_5 and R_6 are MeO, -a-b- is cis- -CH=CH-, -d-e- is $-CH_2-CH_2$ and -f-g- is trans -CH=CH-

90 ml of triethylamine is stirred under an atmosphere of argon and to this are added 7.5 g of a compound of formula V ,in which R_4 is H, R_5 and R_6 are MeO and R_{12} is Me, followed by 10.8 g of the product obtained in step e) above, 0.104 g of palladium diacetate, 0.564 g of tri o-tolyl phosphine and 3.9 g of silver acetate. The mixture is warmed to 80°C with stirring in an oil bath and after 60 hr a further 0.104 g of palladium diacetate and 0.564 g of tri o-tolyl phosphine is added and the reaction continued for a further 48 hr at which time the reaction is complete as judged by thin layer chromatography. The product mixture is concentrated, treated with ethyl acetate and removed from the undissolved residue by suction over Hyflo. The liquid is concentrated and the intermediate trimethylsiloxy/R_{12} is Me product purified by silica gel chromatography (hexane/ethyl acetate 4:1) to give a yield of about 14 g.

5.1 g of NaOH is dissolved in 50 ml of DMSO and 30 ml of water, 7.44 g of the above intermediate product is added and the mixture heated at 100°C for 4 hr with stirring in an oil bath. The mixture is then

cooled, diluted with water, washed 3 times with ether, cooled with ice and adjusted to pH3 with 2N HCl. The product is then extracted 3 times with ethyl acetate, washed twice with saturated brine, dried over Na_2SO_4, concentrated and dried under high vacuum to give 6 g of the title product (m/z: 453 (MH+), 348(60), 329(100), 285(60), 207(60).

g). The compound of formula XIII

5.4 g of the product of step f) above is added to 3.5 l of tetrahydrofuran at room temperature with stirring, 18.7 ml of diethyl azodicarboxylate is added followed by 31.3 g of triphenylphosphine and the mixture warmed to 50°C in an oil bath and allowed to react overnight. The product mixture is concentrated and the residue is purified using 2 Al_2O_3 columns and elution with hexane/ethyl acetate (2:1). Fractions 6-1 from the first column and fractions 7-20 from the second column together yield 5 g of crude product, which is further purified by silica gel chromatography to yield 3.2 g (62%) of the pure resinous intermediate product, being the product corresponding to the compound of formula XIII in which c is in the $CH\text{-}OCH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OCH_3$ protected form.

This intermediate product is converted to the further intermediate product in which c is CHOH by treatment with acid. 5.2 g of the above intermediate product is dissolved in 12 ml of tetrahydrofuran, 10 ml of IN HCl is added and the mixture stirred at 45°C for 24 hr after which it is extracted three times with ethyl acetate and washed with saturated brine and $NaHCO_3$ solutions. The ethyl acetate phases are dried over Na_2SO_4, concentrated and purified by silica gel chromatography (hexane/ethyl acetate 1:1) to give about 3.4 g of the sticky further intermediate product.

2 ml of dichloromethane is stirred at room temperature, 76 µl of oxalyl chloride is added, the mixture cooled to -60°C and a solution of 0.13 ml of DMSO in 1 ml of dichloromethane is added dropwise over 3 min. The resultant evolution of gas is allowed to proceed for 3 min after which a solution of 280 mg of the above further intermediate product in 3 ml of dichloromethane is added dropwise over 5 min with formation of a precipitate. The mixture is stirred for a further 15 min after which 0.56 ml of triethylamine is added over a 5 min period with the resultant precipitation of a paste which is allowed to accumulate at room temperature. The paste is extracted with dichloromethane, the organic phases washed twice with 10% citric acid twice with saturated brine solution, dried over Na_2SO_4, concentrated and purified by silica gel chromatography (dichloromethane) to give the title product of formula XIII in pure form. The pure product has the following characteristics: m. pt.159°C; m/z: 344(M+), 217(80), 189(60), 178(100), 95(60).

Example 7: Preparation of the compound of formula XII

a). The analogue of the compound of formula XII in which c is $CH\text{-}OCH_2\text{-}O\text{-}CH_2\text{-}CH_2\text{-}OCH_3$

2.9 g of Iodine and 0.55 g of magnesium are added to 20 ml of diethyl ether and stirred until the exothermic reaction is completed and the brown colour has disappeared. By means of a syringe 11 g of the resultant solution is removed and added dropwise over a period of 10 min to a solution in benzene of 1.64 g of the first intermediate product of step g) of Example 6 above. A yellow precipitate forms and the mixture is warmed to 80°C in an oil bath and stirred for 1 1/2 hr at this temperature and then stirred overnight at room temperature. The product is then extracted twice with ethyl acetate, washed with $NaHCO_3$ and saturated brine, dried over Na_2SO_4, concentrated and purified by silica gel chromatography (hexane/ethyl acetate 7:3) to give 1.6 g of the title product in pure form.

b). The analogue of the compound of formula XII in which c is CHOH

510 mg of the product of step a) of this Example is dissolved in 12 ml of tetrahydrofuran, 10 ml of 1 N HCl is added and the mixture stirred at 45°C for 24 hr. The product mixture is extracted three times with ethyl acetate, and washed with saturated brine and $NaHCO_3$. The ethyl acetate phases are dried over Na_2SO_4, concentrated and purified by silica gel chromatography (hexane/ethyl acetate 1:1) to give 330 mg of the sticky title product (m/z: 332(M+), 314(10), 203(75), 190(55), 164(60).

c). The compound of formula XII

280 mg of the product of step b) is dissolved in 10 ml of dichloromethane with stirring at room temperature, 475 mg of pyridinium dichromate is added and the mixture is stirred overnight at room temperature. After this a further 158 mg of pyridinium dichromate is added and the mixture is stirred again overnight and for a further 24 hr at room temperature. The resultant product solution is separated from the solid byproducts by filtration through Hyflo and purified chromatographically (elution with dichloromethane) to yield the title product in pure form (m. pt. 137°C; m/z: 330(M+), 312(20), 202(100), 175(75))

Example 8: The compound of formula I in which R_4 is H, R_5 and R_6 are OMe, -a-b- is cis -CH=CH-, c is C=O and -d-e- and -f-g- are $-CH_2-CH_2-$

a). 4-dimethyl,t-butyl-silyloxy-pent-1-yne

17.8 g of (±)-4-pentyn-2-ol is dissolved in 150 ml of acetonitrile with stirring at room temperature, 15.8 g of imidazole followed by 35 g of dimethyl,t butyl-silyl chloride are added giving rise to an exothermic reaction whereby all components of the reaction mixture initially go into solution but afterwards a precipitate immediately forms. The mixture is stirred overnight at room temperature after which it is suction filtered, the liquid fraction concentrated and a further 2.9 g of imidazole and 6.3 g of dimethyl,t butyl-silyl chloride are added. After 3 hr the reaction mixture is again suction filtered, the liquid fraction concentrated subjected to fractional distillation. The title product is in the first three fractions which are combined and the product purified by distillation at reduced pressure (12 mm Hg) at 64-68°C.

b). The product of formula VII in which R_4 is H, R_5 and R_6 are OMe and R_{12} is Me

14.6 g of the corresponding compound of formula V, 14.6 g of the product of step a) of this Example, 0.3 g of palladium diacetate, 1.1 g of tri o-tolyl phosphine and 7.6 g of silver acetate are added to 60 ml of tetrahydrofuran and the mixture warmed with stirring to 70°C in an oil bath. After 48 hr, at which time the reaction is about 70% completed as judged by thin layer chromatography, a further 0.3 g of palladium diacetate and 1.1 g of tri o-tolyl phosphine are added and the mixture warmed over the weekend at 70°C. The mixture is then suction filtered over Hyflo, concentrated under high vacuum and purified by silica gel chromatography (hexane/ethyl acetate 4:1) to give a first intermediate product, i.e. an analogue of the corresponding compound of formula VII in which R_6 = OMe, R_5 = OMe, R_4 = H, R_{12} = Me and the aldehyde group of the C_6 side chain is a dimethyl,t-butyl-silyl-protected OH group.

6.7 g of this first intermediate product is dissolved in 100 ml of methanol, 500 mg of palladium/charcoal catalyst containing 10% palladium (Pd/C 10%) is added and the mixture hydrogenated under stirring for 48 hr (350 ml of H_2 being taken up after the first 24 hr). The reaction mixture is then suction filtered over Hyflo and concentrated; though at this stage it is found that the C_6 side chain double bond is only 30% reduced as judged by NMR. The reaction product is, therefore again taken up in 150 ml of methanol, 500 mg of the Pd/C 10% catalyst is added and the mixture hydrogenated over the weekend at room temperature. The resultant reaction mixture is suction filtered over Hyflo, concentrated and a second intermediate product, i.e. an analogue of the corresponding compound of formula VII in which the aldehyde group of the C_6 side chain is an OH group, is separated from the corresponding siloxy-protected compound by silica gel chromatography (hexane/ethyl acetate 2:1) in a yield of 4.9 g.

1.2 ml of oxalyl chloride is added to 30 ml of dichloromethane stirred at room temperature, the mixture cooled to -60°C and 1.9 ml of DMSO in 7 ml of dichloromethane added dropwise over a 5 min period after which the mixture is stirred for a further 3 min and a solution of 3.5 g of the second intermediate product of this example in 20 ml of dichloromethane is added dropwise over a 10 min period. A precipitate forms, the mixture is stirred for a further 20 min, concentrated and 8.2 ml of triethylamine is added over a 5 min period. A thick paste precipitate forms and is allowed to accumulate at room temperature for 1.5 hr. The solid product is extracted with dichloromethane, washed twice with 10% citric acid solution, once each with saturated brine and $NaHCO_3$, dried over Na_2SO_4 and concentrated to give 3.6 g of the title product in the form of gum.

c). The product of formula IV in which in which R_4 is H, R_5 and R_6 are OMe, -a-b- is cis -CH=CH-, -d-e- and -f-g- are $-OH_2-OH_2-$ and R_{11} is H

0.39 g of the product of step a) of this Example is added to 5 ml of tetrahydrofuran under an atmosphere of nitrogen, 1.2 ml of butyl lithium is added dropwise over a 2 min period at -70°C. After 1 hr a solution of 0.5 g of the final product of step b) of this Example in 4 ml of tetrahydrofuran is added dropwise over a 5 min period, the mixture stirred at -70°C for 1.5 hr and then allowed to warm to room temperature. At this point the reaction is complete as judged by thin layer chromatography. Whilst cooling with ice, the product is washed with 10% citric acid solution, extracted three times with ethyl acetate, washed with saturated brine and $NaHCO_3$, dried over Na_2SO_4 and concentrated to give 0.87 g of a resinous first intermediate product, i.e. an analogue of the corresponding compound of formula IV in which the COOH substituent is in the form of the methoxy ester, the bond corresponding to the -a-b- bond of the compound of formula I is an acetylene bond and the OH substituent on the C_{10} carbon atom of the C_{11} side chain is t-butyl-silyl-protected.

3.1 g of this first intermediate product is dissolved in 50 ml of pyridine, 200 mg of 10% Pd/$BaSO_4$ is added and the mixture hydrogenated at room temperature with magnetic stirring. After 4 hr greater than the theoretical hydrogen requirement (240 ml) has been taken up. The resultant product is then sampled, concentrated, taken up in dichloromethane and washed with 10% citric acid solution (at this point the re-

action is judged to be complete by NMR). The product is separated from the catalyst by suction filtration over Hyflo, concentrated, taken up in ethyl acetate, washed with saturated brine, dried over Na_2SO_4 and concentrated to give 3.05 g of a resinous second intermediate product, i.e. the analogue of the first intermediate product of this step in which the bond corresponding to the -a-b- bond of the compound of formula I is cis -CH=CH-.

By analogy with step f) of Example 6 the second intermediate product of this step is converted into the title product (m/z: 367(MH+), 349(40), 331(100), 305(90), 191(100)).

d). The compound of formula I in which R_4 is H, R_5 and R_6 are OMe, -a-b- is cis -CH=CH- c is C=O and -d-e- and -f-g- are $-CH_2-CH_2-$

1.7 g of the final product of step c) above is dissolved with stirring in 1.8 l of acetonitrile, 5.9 g of 2-chloropyridine iodide and 6.5 ml of triethylamine are added and the mixture is warmed over the weekend to 50°C in an oil bath with stirring. The product is then concentrated, taken up in ethyl acetate, washed with 10% citric acid solution $NaHCO_3$ and saturated brine, dried over Na_2SO_4 and concentrated to give 1.6 g of a crude intermediate product (i.e. the analogue of the title product in which c is CHOH) in the form of a brown resin. This crude product is purified by silica gel chromatography (hexane/ethyl acetate 3:2), using dichloromethane to dissolve the intermediate product.

To 2 ml of dichloromethane at room temperature is added 5 µl of oxalyl chloride, the mixture cooled to -70°C and a solution of 93 µlof DMSO in 1 ml of dichloromethane is added dropwise over 3 min with stirring. The mixture is stirred for a further 5 min after which a solution of 0.2 g of the intermediate product of this step is added dropwise over 5 min. A precipitate forms, the mixture is stirred for a further 15 min, 0.4 ml of triethylamine is added dropwise over 5 min, the mixture stirred for 1 hr at -70°C and then allowed to warm to room temperature over 1 hr. The resultant product mixture is diluted with dichloromethane, washed twice with 10% citric acid solution, once with saturated brine, dried over Na_2SO_4, concentrated and purified by silica gel chromatography and recrystallisation to give about 100 mg of pure product (m. pt. 110°C).

Example 9: The compound of formula I in which R_4 and R_6 are H, R_5 is OMe, -a-b- is trans -CH=CH- c is C=O and -d-e- and -f-g- are $-CH_2-CH_2-$

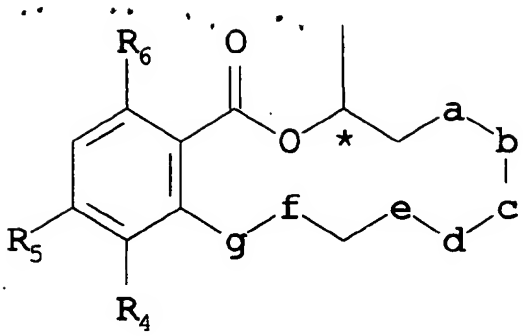
35 mg of magnesium is stirred with 1 ml of diethylether and 1 ml of benzene and 0.18 g of iodine is added, the dark brown colour dissipating after 1.5 hr. The liquid phase of the resultant mixture is added to a solution of 50 mg of the final product of Example 6 in 2 ml of benzene with the resultant formation of a precipitate. The mixture is stirred at 60°C for 2.5 hr, the product taken up in ethyl acetate, washed with 1N HCl saturated $NaHCO_3$ and saturated brine, dried over Na_2SO_4, concentrated and purified by silica gel chromatography (toluene/methanol 98:2). The product is further purified on a silica gel column (hexane/ethyl acetate 3:2) to give about 30 mg of the pure title product in the form of an oil (m/z: 333(MH+), 315(80), 265(100), 177(90).

Example 10: The compound of formula I in which R_4 is H, R_5 and R_6 are OMe, -a-b- is trans -CH=CH- c is C=O and -d-e- and -f-g- are $-CH_2-CH_2-$

By analogy with Examples 3 and 9 the title product is prepared form the corresponding product in which -a-b- is cis -CH=CH-, i.e. the final product of Example by isomerisation of the -a-b- bond. The title product is obtained in the form of an oil (m/z: 346(M+), 205(50), 191(100), 178(40), 152(40, 95(55).

Claims

1. A compound of Formula I



I

in which R_4 and R_6 are the same or different and are H, OH, C_{1-4} alkoxy or C_{1-4}alkyl COO-,
R_5 is OH, C_{1-4}alkoxy or C_{1-4}alkyl COO-,
one of -a-b- or -d-e- is $-CHR_7-CHR_8-$ and the other is cis- or trans- $-CR_7=CR_8-$, wherein R_7 and R_8 are the same or different and are H, OH, C_{1-4} alkoxy or C_{1-4}alkyl COO-,
c is CH-OH or C=O and
-f-g- is $-CH_2-CH_2-$ or cis- or trans- -CH=CH-
provided that, when R_4 is H, R_6 is OH and -f-g- is trans- -CH=CH-; 1. R_5 is not OH when -a-b- is $-CH_2-CH_2-$, c is C=O and -d-e- is $-CH_2-CH_2-$, or 2. R_5 is not methoxy when -a-b- is $-CH_2-CH_2-$ or cis- -CH=CH- and c is C=O or CH-OH and -d-e- is -CHOH-CHOH-,
in free form or base salt form or in the form of a physiologically-hydrolysable and -acceptable ester and wherein the asymmetric carbon marked * and the atoms a and/or b or d and/or e, when these are asymmetric carbon atoms have the R- or S-configuration or the compound comprises any mixture of the optical isomers thereof.

2. A compound according to claim 1 in which
R_4 and R_6 are the same or different and are H, -OH, MeO- or Me-COO-, R_5 is -OH, MeO- or MeCOO-, -a-b- is cis- or trans- $-CR_7'=CR_8'-$, wherein R_7' and R_8' are the same or different and are H, OH, MeO- or Me-COO- and -d-e- is $-CHR_7'-CHR_8'-$, wherein R_7' and R_8' are as defined.

3. A compound according to claim 2, in which
R_4 is H or MeO, R_5 is MeO, R_6 is OH or MeO, -a-b- is cis- or trans- -CH=CH-, -d-e- is $-CH_2-CH_2-$ or -CHOH-CHOH- and -f-g- is trans- -CH=CH-.

4. A compound of formula 1 in which
R_4 is H or methoxy, R_5 is methoxy, R_6 is OH, -a-b- is cis- or trans- -CH=CH-, c is CHOH or C=O, -d-e- is -CHOH-CHOH- and -f-g- is trans- -CH=CH-; provided that when -a-b- is cis -OH=OH-, then R_4 is methoxy and c is C=O in free form or base salt form or in the form of a physiologically-hydrolysable and -acceptable ester.

5. A compound according to any of the preceding claims, in which a-b is trans- -CH=CH-.

6. A compound according to any of the preceding claims, in which the asymmetric carbon atoms all have the S-configuration.

7. A compound of formula I', I'', X, XI, XII, or XIII as hereinbefore defined.

8. A process for the preparation of the compound of formula I' comprising the steps of cultivating an 87-250904-F1-producing fungal strain and isolating the metabolite 87-250904-F1 which is formed.

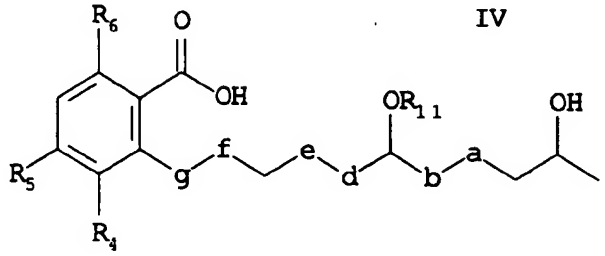
9. A process according to Claim 8 in which the fungal strain is NRRL 18919.

10. A pure culture of fungal strain NRRL 18919.

11. A fermentation broth which is obtained by cultivation of the fungal strain NRRL 18919.

12. A process for the preparation of a compound of formula I in which a-b is trans- -CH=CH- comprising the step of isomerization of the corresponding cis- isomer under mild alkaline conditions.

13. A process for the preparation of a compound of formula I, in which c is CHOH, which comprises cyclising a compound of formula IV

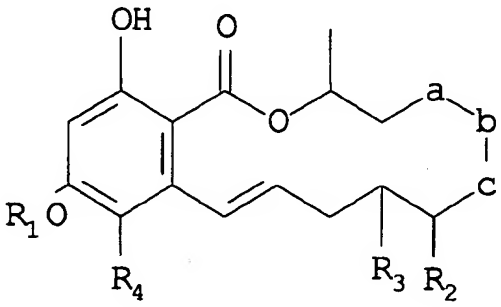


IV

wherein R_4, R_5, R_6, -a-b-, -d-e- and -f-g- are as defined in claim 1 with the exception that any OH substituents on -a-b- or -d-e- are in suitably protected form, and R_{11} is H or an OH protecting group; and removing any OH protecting groups therefrom.

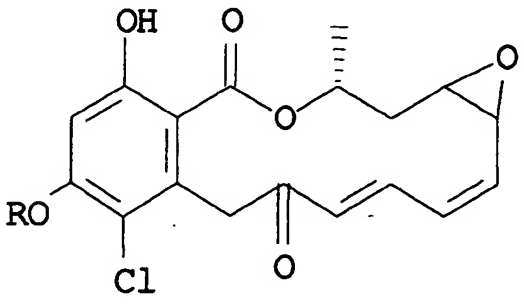
14. A process for the preparation of a compound of formula I, in which c is C=O comprising oxidising the c CHOH group of a compound of formula I wherein c is CHOH.

15. A method for the treatment of disorders with an aetiology associated with or comprising excessive cytokine release, particularly IL-1β release, such as rheumatoid arthritis, osteoarthritis, septic shock, psoriasis, atherosclerosis, inflammatory bowel disease, Crohn's disease and asthma, in a subject in need thereof, which method comprises administering to said subject an effective amount of
    a) a compound of Claim 1; or
    b) a compound of formula II



II

in which:
R_1 is H or methyl;
R_2 and R_3 are H or OH,
R_4 is H or methoxy;
-a-b- is $-CH_2-CH_2-$ or -CH=CH-
and c is C=O or CH-OH (other than a compound of Claim 1); or
    c) a compound of formula III



III

in which R is H or methyl
in free or base salt form, or a physiologically-hydrolysable and -acceptable ester thereof.

16. A compound of formula II, stated in Claim 15, in which:
R_1 is methyl;
R_2 and R_3 are H or OH,
R_4 is H or methoxy;
-a-b- is $-CH_2-CH_2-$ or cis- -CH=CH-;
and c is C=O or CH-OH;
in free base or salt form, or a physiologically-hydrolysable and -acceptable ester thereof for use as a pharmaceutical.

17. A pharmaceutical composition comprising a compound according to claim 1.

18. A pharmaceutical composition comprising a compound of formula II, stated in Claim 15, in which:
R_1 is methyl; R_2 and R_3 are H or OH; R_4 is H or methoxy;-a-b- is $-CH_2-CH_2-$ or cis- -CH=CH-; and c is C=O or CH-OH; in free base or salt form, or a physiologically-hydrolysable and -acceptable ester thereof.

FIG. 1

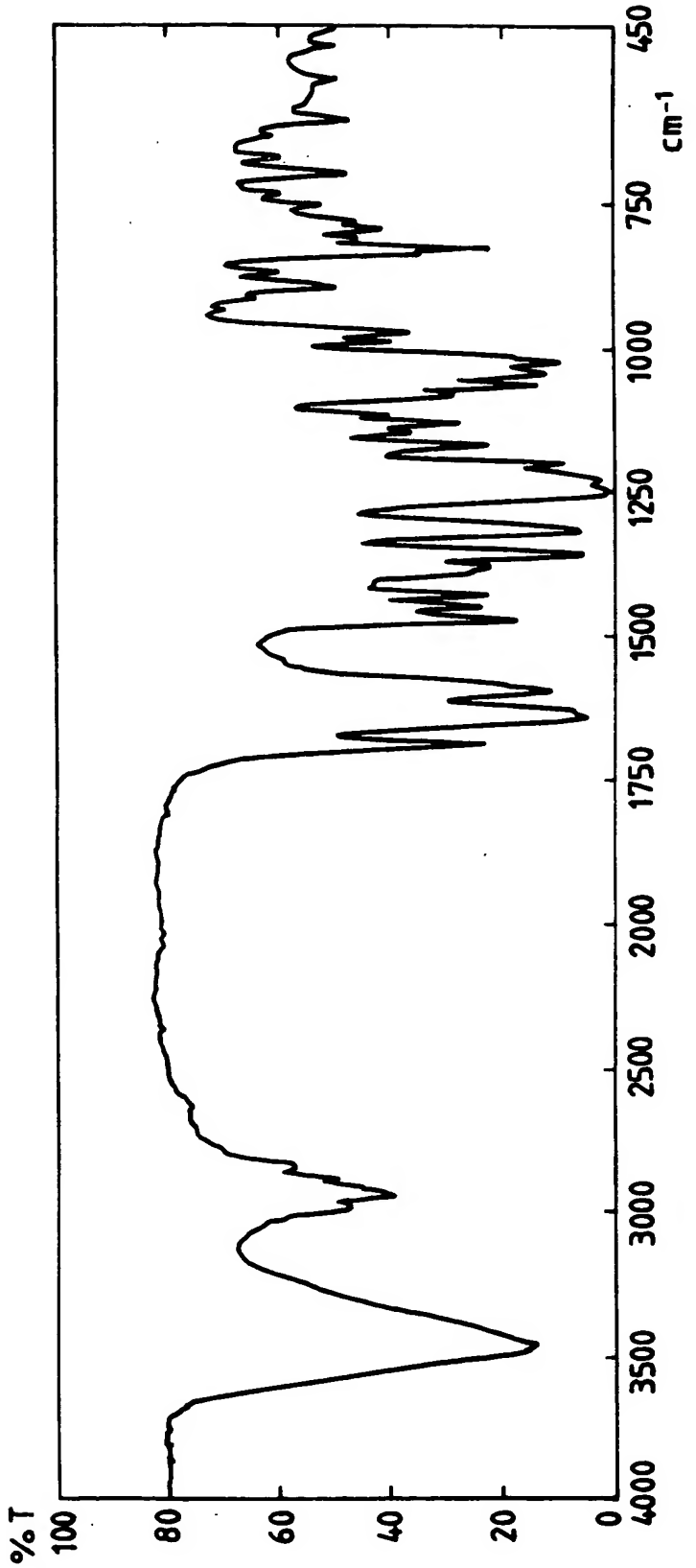
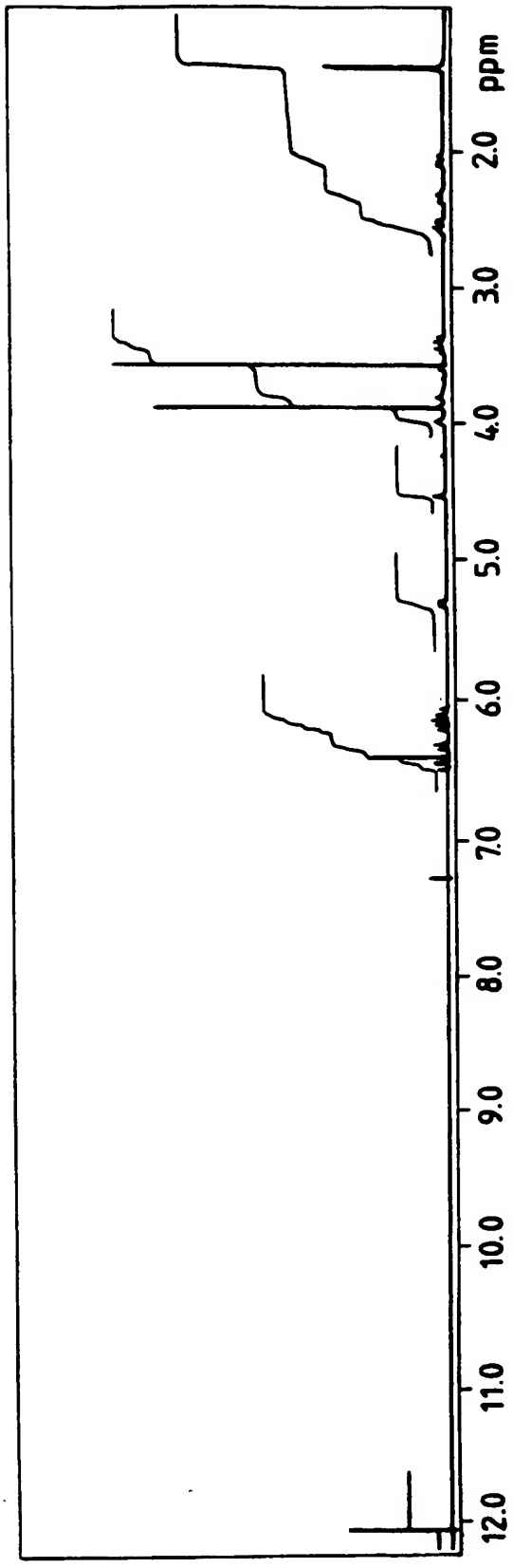

%T
100
80
60
40
20
0
4000
3500
3000
2500
2000
1750
1500
1250
1000
750
450
cm-1

FIG. 2



European Patent Office

PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 93 81 0835

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	US-A-3 196 019 (F.N. ANDREWS ET AL.) * column 3 - column 8 * ---	1	C07D313/00 A61K31/365 C12P17/08
A	HELVETICA CHIMICA ACTA vol. 55, no. 8 , 1972 , BASEL CH pages 3030 - 3048 G. BOLLIGER, CH. TAMM '306. VIER NEUE METABOLITE VON GIBERELLA ZEAE.' * page 3030 - page 3032; example 8 * ---	1,4,5,7	
A	JOURNAL OF ORGANIC CHEMISTRY. vol. 43, no. 12 , 1978 , EASTON US pages 2339 - 2343 G. ELLESTAD ET AL. 'NEW ZEARALENONE RELATED MACROLIDES AND ISOCOUMARINS FROM AN UNIDENTIFIED FUNGUS.' * page 2339 - page 2343 * -----	1,4,7-11	

TECHNICAL FIELDS SEARCHED (Int.Cl.5)

C07D
C12P

INCOMPLETE SEARCH

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
THE HAGUE	23 March 1994	Francois, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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EP 93 81 0835

-C-

Remark: Although claim 15 is directed to a method of treatment of the human/animal body (Art. 52(4) EPC) the search has been carried out and based on the alleged effects of the compound/composition

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(54) Title: METHODS OF MODULATING HAIR GROWTH

(57) Abstract: The invention features methods of promoting hair growth in a subject. The methods include inducing or mimicking the effects of Wnt promoted signal transduction, e.g., by increasing the level of Wnt protein or administering an agent which mimics an effect of Wnt promoted signal transduction, e.g., by administering lithium chloride. Methods of inhibiting hair growth are also provided.

WO 01/74164 A1

METHODS OF MODULATING HAIR GROWTH

Related Applications

This application claims the benefit of U.S. provisional application Serial No. 60/261,690, filed January 12, 2001, and U.S. provisional application Serial No. 60/193,771, filed March 31, 2000, both of which are incorporated herein by reference.

Background of the Invention

The hair follicle undergoes a cycle of hair growth (anagen) followed by regression (catagen), and quiescence (telogen) until a new hair shaft is generated in the existing follicle during the subsequent anagen phase. Hardy et al. (1992) *Trends in Genetics* 8:55-61. The hair shaft is derived from the epithelial matrix cells at the base of the follicle, but a cluster of dermal cells ensheathed by the matrix cells, known as the dermal papilla (DP), is thought to supply inductive signals required for hair outgrowth. Reciprocal signaling from the epidermis is required for the formation of the dermal papilla and may also explain the coordinated morphological changes in epithelial and dermal components of the follicle observed during the hair cycle.

Summary of the Invention

The invention is based, in part, on the discovery that increasing Wnt protein levels can positively regulate the ability of dermal papilla (DP) cells to promote hair growth. It was found that co-culture of Wnt expressing cells with DP cells maintains hair inductivity. In addition, it was found that agents, such as inhibitors of GSK3β kinase, e.g., lithium chloride or similar small ions, which can mimic an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, can regulate the ability of DP cells to promoter hair growth.

Accordingly, in one aspect, the invention features a method of promoting hair growth in a subject. The method includes inducing or mimicking the effects of Wnt promoted signal transduction, e.g., by increasing the level of Wnt protein or administering an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, to thereby promote hair growth.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, Wnt is increased by administering an agent which increases the level of Wnt protein production and/or activity. An agent which increases the level of Wnt protein and/or which mimics an effect of Wnt promoted signal transduction can be one or more of: a Wnt polypeptide or a functional fragment or analog thereof; a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof; an agent which increases Wnt nucleic acid expression, e.g., a small molecule which binds to the promoter region of Wnt; an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. Examples of agents which can mimic an effect of Wnt promoted signal transduction include: an inhibitor of GSK3β kinase, e.g., lithium chloride or similar small ions; agents which bind Frizzled (Frz) (a cell surface receptor) and mimic Wnt binding, e.g., anti-Frizzled antibodies, or other naturally or non-naturally occurring Frizzled binding ligands.

In a preferred embodiment, Wnt is increased by administering, e.g., introducing, a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof, into a particular cell, e.g., an epidermal cell or a DP cell, in the subject. The nucleotide sequence can be a genomic sequence or a cDNA sequence. The nucleotide sequence can include: a Wnt coding region; a promoter sequence, e.g., a promoter sequence from a Wnt gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR from a Wnt gene or from another gene, a 3' UTR, e.g., a 3' UTR from a Wnt gene or from another gene; a polyadenylation site; an insulator sequence.

In another preferred embodiment, the level of Wnt protein is increased by increasing the level of expression of an endogenous Wnt gene, e.g., by increasing transcription of the Wnt gene. In a preferred embodiment, transcription of the Wnt gene is increased by: altering the regulatory sequences of the endogenous Wnt gene, e.g., by the addition of a positive regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); the deletion of a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and/or replacement of the endogenous regulatory sequence, or

elements therein, with that of another gene, thereby allowing the coding region of the Wnt gene to be transcribed more efficiently.

In another embodiment, the method can include introducing a cell into a subject, e.g., a cell expressing Wnt. In a preferred embodiment, the cell expresses a Wnt protein, e.g., a Wnt 3, Wnt 4, or Wnt 7, or a fragment or an analog thereof. In another preferred embodiment, the cell has been genetically modified to cause the expression of Wnt, e.g., the cell has been genetically modified to express a Wnt protein, or a fragment or an analog thereof, or the cell has been genetically modified to introduce a nucleic acid sequence, e.g., a regulatory sequence, e.g., a promoter or an enhancer, that causes or increases the expression of the endogenous Wnt. In a preferred embodiment, the promoter of the endogenous Wnt gene has been replaced by another promoter, e.g., by a promoter from another gene. The cell can be an autologous, allogeneic, or xenogeneic cell, but is preferably autologous. The autologous cell is preferably from a subject characterized with hair loss. The manipulated cell can be any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a lymphocyte, a bone marrow cell, and a muscle cell. Preferably the cell is an epithelial cell, e.g., an epidermal cell, a hair follicle cell, a dermal papilla cell. The cell can be introduced into a subject to increase Wnt activity.

In a preferred embodiment, the level of Wnt, e.g., Wnt 3, Wnt 4, or Wnt 7, is increased over a sustained period of time, e.g., a period equal to or greater than 2, 10, 14, 30, 60, 90, or 180 days. E.g., a cell expressing a Wnt protein, fragment, or analog can be supplied, e.g., by any method described herein, whereby Wnt is released over a sustained period of time, e.g., a period equal to or greater than 2, 10, 14, 30, 60, 90, or 180 days.

In a preferred embodiment, the agent which increases the level of Wnt protein or mimics an effect of Wnt promoted signal transduction is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered:

by continuous administration, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt protein level or Wnt promoted signal transduction is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, hair growth is promoted on: the subject's scalp; the subject's face, e.g., beard and/or mustache facial hair growth is promoted.

In a preferred embodiment, the subject has an insufficient amount of hair or an insufficient rate of hair growth. In a preferred embodiment, the subject suffers from genetic pattern baldness; suffers from a hormonal disorder which decreases hair growth; has received a treatment, e.g., radiation, or chemotherapy, or a drug which inhibits hair growth; or has had a surgical procedure, e.g., skin graft, which is in need of hair growth.

In another aspect, the invention features a method of inhibiting hair growth in a subject. The method includes inhibiting the level of Wnt protein or inhibiting an effect of Wnt promoted signal transduction, in the subject.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, Wnt is inhibited by administering an agent which inhibits Wnt protein production levels and/or is a Wnt antagonist. An agent which inhibits Wnt can be one or more of: a Frizzled protein or Wnt binding portion thereof; a Wnt nucleic acid molecule which can bind to a cellular Wnt nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein, e.g., an antisense molecule or Wnt ribozyme; an antibody that specifically binds to Wnt protein, e.g., an antibody that disrupts Wnt's ability to bind to its natural cellular target, e.g., disrupts Wnt's ability to bind to Frizzled; an antibody that specifically binds to Frizzled, e.g., an antibody that disrupts Frizzled's ability to bind to Wnt; a mutated inactive Wnt protein or fragment which binds to Frizzled but does not activate the Wnt signaling pathway; an agent which decreases Wnt gene expression, e.g., a small molecule which binds the promoter of Wnt.

In another preferred embodiment, Wnt is inhibited by decreasing the level of expression of an endogenous Wnt gene, e.g., by decreasing transcription of the Wnt gene. In a preferred embodiment, transcription of the Wnt gene can be decreased by: altering the regulatory sequences of the endogenous Wnt gene, e.g., by the addition of a negative regulatory sequence (such as a DNA-binding site for a transcriptional repressor).

In a preferred embodiment, the agent which decreases the level of Wnt protein of inhibits an effect of Wnt promoted signal transduction is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered: by continuous administration, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt protein level or Wnt promoted signal transduction is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, hair growth is inhibited on: the subject's scalp; the subject's face, e.g., beard and/or mustache facial hair growth or eyebrow growth is inhibited; the subject's body hair growth is inhibited, e.g., hair growth is inhibited on the subject's back, legs, chest, armpits.

In another aspect, the invention features a method of promoting hair growth in a subject. The method includes activating or increasing activation of the Wnt-β-catenin signaling pathway.

In a preferred embodiment, activation of the Wnt-β-catenin pathway is increased by administering an agent which increases the level of Wnt protein production and/or which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. An agent which increases the level of Wnt protein and/or which mimics an effect of Wnt

promoted signal transduction can be one or more of: a Wnt polypeptide or a functional fragment or analog thereof as described herein; a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof as described herein; an agent which increases Wnt nucleic acid expression, e.g., a small molecule which binds to the promoter region of Wnt; an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. Examples of agents which can mimic Wnt promoted signal transduction include inhibitors of GSK3β, e.g., lithium chloride or similar small ions, agents which bind Frizzled and mimic Wnt binding, e.g., anti-Frizzled antibodies, or other naturally or non-naturally occurring Frizzled binding ligands. In other embodiments, the level of Wnt protein can be increased by increasing the level of expression of an endogenous Wnt gene, e.g., by increasing transcription of the Wnt gene, as described herein. In another preferred embodiment, activation of the Wnt-β-catenin pathway is increased by administering: an agent which increases β-catenin protein production or activity, e.g., an agent which decreases phosphorylation of β-catenin and/or which increases β-catenin accumulation, e.g., a β-catenin polypeptide or a fragment or analog thereof, a nucleic acid sequence encoding a β-catenin polypeptide or fragments or analogs thereof; an agent which increases LEF-1 protein production and/or activity, e.g., an LEF-1 polypeptide or a fragment or analog thereof, a nucleic acid sequence encoding an LEF-1 polypeptide or fragments or analogs thereof.

In another preferred embodiment, the method can include introducing a cell, e.g., a cell which expresses and preferably secretes a protein involved in the activation of the Wnt-β-catenin signaling pathway, into a subject. In a preferred embodiment, the cell has been genetically modified to express: a Wnt protein, or a fragment or an analog thereof; a β-catenin protein, or fragment or analog thereof; an LEF-1 protein, or a fragment or analog thereof. In a preferred embodiment, the cell expresses a Wnt protein, e.g., a Wnt 3, Wnt 4, or Wnt 7, or a fragment or an analog thereof. In another preferred embodiment, the cell has been genetically modified to cause the expression of Wnt, e.g., the cell has been genetically modified to express a Wnt protein, or a fragment or an analog thereof, or the cell has been genetically modified to introduce a nucleic acid sequence, e.g., a regulatory sequence, e.g., a promoter or an enhancer, that causes or increases the expression of the endogenous Wnt. In a preferred embodiment, the promoter of the endogenous Wnt gene has been replaced by

another promoter, e.g., by a promoter from another gene. The cell can be an autologous, allogeneic, or xenogeneic cell, but is preferably autologous. The autologous cell is preferably from a subject characterized with hair loss. The manipulated cell can be any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell. Preferably the cell is an epithelial cell, e.g., an epidermal cell, a hair follicle cell, a dermal papilla cell. The cell can be introduced into a subject to increase Wnt activity.

In a preferred embodiment, the level of Wnt, e.g., Wnt 3, Wnt 4, or Wnt 7, is increased over a sustained period of time, e.g., a period equal to or greater than 2, 10, 14, 30, 60, 90, or 180 days. E.g., a cell expressing a Wnt protein, fragment, or analog can be supplied, e.g., by any method described herein, whereby Wnt is released over a sustained period of time, e.g., a period equal to or greater than 2, 10, 14, 30, 60, 90, or 180 days. The cell can be introduced into a subject, e.g., to increase the level of the protein involved in activation of the Wnt-β-catenin signaling pathway.

In a preferred embodiment, the agent is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered: by continuous administration, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt-β-catenin signaling pathway is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, hair growth is promoted on: the subject's scalp; the subject's face, e.g., beard and/or mustache facial hair growth is promoted.

In a preferred embodiment, the subject has an insufficient amount of hair or an insufficient rate of hair growth. In a preferred embodiment, the subject suffers from genetic pattern baldness; suffers from a hormonal disorder which decreases hair growth; has received a treatment, e.g., radiation, or chemotherapy, or a drug which inhibits hair growth; or has had a surgical procedure, e.g., skin graft, which is in need of hair growth.

In another aspect, the invention features a method of inhibiting hair growth in a subject. The method includes inhibiting activation of the Wnt-β-catenin signaling pathway.

In a preferred embodiment, activation of the Wnt-β-catenin pathway is inhibited by administering an agent which decreases the level of Wnt protein production and/or decreases an effect of Wnt promoted signal transduction. An agent which inhibits the level of Wnt protein or is a Wnt antagonist can be one or more of: a Frizzled protein or Wnt binding portion thereof; a Wnt nucleic acid molecule which can bind to a cellular Wnt nucleic acid sequence, e.g., mRNA, and inhibit expression of the protein, e.g., an antisense molecule or Wnt ribozyme; an antibody that specifically binds to Wnt protein, e.g., an antibody that disrupts Wnt's ability to bind to its natural cellular target, e.g., disrupts Wnt's ability to bind to a Frizzledreceptor protein; an antibody that specifically binds to Frizzled, e.g., an antibody that disrupts a Frizzled's ability to bind to Wnt; a mutated inactive Wnt protein or fragment which binds to Frizzled but does not activate the Wnt signaling pathway; an agent which decreases Wnt gene expression, e.g., a small molecule which binds the promoter of Wnt; an agent which decreases an activity of Wnt, e.g., an agent which increases phosphorylation of β-catenin. In other embodiments, the level of Wnt protein can be inhibited by decreasing the level of expression of an endogenous Wnt gene, e.g., by decreasing transcription of the Wnt gene, as described herein. In another preferred embodiment, activation of the Wnt-β-catenin pathway is inhibited by administering: an agent which inhibits β-catenin protein production or inhibits an effect of Wnt promoted signal transduction, e.g., an agent which increases phosphorylation of β-catenin and/or which decreases β-catenin accumulation; an agent which inhibits LEF-1 protein production and/or activity.

In a preferred embodiment, the agent is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered: by continuous administration, e.g., the agent is administered with sufficient frequency such that the effect

on the Wnt-β-catenin signaling pathway is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, hair growth is inhibited on: the subject's scalp; the subject's face, e.g., beard and/or mustache facial hair growth or eyebrow growth is inhibited; the subject's body hair growth is inhibited, e.g., hair growth is inhibited on the subject's back, legs, chest, armpits.

In another aspect, the invention features a method of evaluating the status of hair growth/hair loss in a subject. The method includes evaluating, e.g., detecting, the presence or absence of a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, misexpression of the Wnt gene.

In one embodiment, the method includes evaluating whether a subject is at risk for hair loss. The method includes evaluating, e.g., detecting, a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, underexpression of the Wnt gene, to thereby determine if a subject is at risk for hair loss.

In a preferred embodiment, the Wnt gene or protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In preferred embodiment, the method includes evaluating in a sample of cells from the subject for the presence or absence of a genetic lesion, e.g., a mutation in the gene encoding a Wnt protein. The presence of a genetic lesion is indicative of a risk of hair loss in a subject. The cell sample can be of any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a lymphocyte, a bone marrow cell, and a muscle cell.

In another preferred embodiment, the method includes evaluating in a sample of cells, e.g., a sample of epidermal cells from the hair follicle of a subject, for the expression levels of the Wnt to determine underexpression. Underexpression of Wnt is indicative of a risk of hair loss.

In a preferred embodiment, the genetic lesions is evaluated by contacting the sample with a nucleic acid probe capable of hybridizing to Wnt mRNA, e.g., a labeled probe. In

another preferred embodiment, expression of Wnt is evaluated with an antibody capable of binding to Wnt protein, e.g., a labeled antibody.

In another embodiment, the method includes evaluating hair growth in a subject. The method includes evaluating, e.g., detecting, absence or presence of a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, overexpression of the Wnt gene, to thereby evaluate whether hair growth is likely in a subject.

In a preferred embodiment, the Wnt gene or protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, the method includes evaluating in a sample of cells from the subject for the presence or absence of a genetic lesion, e.g., a mutation in the gene encoding a Wnt protein. The absence of a genetic lesion is indicative of a potential for hair growth. The cell sample can be of any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell, an endothelial cell, a glial cell, a neural cell, a lymphocyte, a bone marrow cell, and a muscle cell.

In another preferred embodiment, the method includes evaluating in a sample of cells, e.g., a sample of epidermal cells from the hair follicle of a subject, for the expression levels of Wnt to determine overexpression. Overexpression of Wnt is indicative of a potential for hair growth.

In a preferred embodiment, the genetic lesions is evaluated by contacting the sample with a nucleic acid probe capable of hybridizing to Wnt mRNA, e.g., a labeled probe. In another preferred embodiment, expression of Wnt is evaluated with an antibody capable of binding to Wnt protein, e.g., a labeled antibody.

In another aspect, the invention features a method of evaluating the ability of an epidermal cell to promote hair growth or hair loss in a subject. The method includes evaluating, e.g., detecting, the presence or absence of a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, misexpression of the Wnt gene.

In a preferred embodiment, the ability of an epidermal cell to promote hair growth or hair loss is evaluated *in vitro*.

In one embodiment, the method includes evaluating the ability of an epidermal cell to promote hair loss. The method includes evaluating, e.g., detecting, a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, underexpression of the Wnt gene.

In a preferred embodiment, the Wnt gene or protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt 3, most preferably, Wnt3a.

In preferred embodiment, the method includes evaluating in a sample of epidermal cells from the subject for the presence or absence of a genetic lesion, e.g., a mutation in the gene encoding a Wnt protein. The presence of a genetic lesion is indicative of a risk of hair loss in a subject.

In another preferred embodiment, the method includes evaluating in a sample of epidermal cells, for the expression levels of the Wnt to determine underexpression. Underexpression of Wnt is indicative of a risk of hair loss.

In a preferred embodiment, the genetic lesions is evaluated by contacting the sample with a nucleic acid probe capable of hybridizing to Wnt mRNA, e.g., a labeled probe. In another preferred embodiment, expression of Wnt is evaluated with an antibody capable of binding to Wnt protein, e.g., a labeled antibody.

In another embodiment, the method includes evaluating the ability of an epidermal cell to promote hair growth. The method includes evaluating, e.g., detecting, absence or presence of a genetic lesion in a Wnt gene, or evaluating, e.g., detecting, overexpression of the Wnt gene.

In a preferred embodiment, the Wnt gene or protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably, Wnt3a.

In a preferred embodiment, the method includes evaluating in a sample of epidermal cells from the subject for the presence or absence of a genetic lesion, e.g., a mutation in the gene encoding a Wnt protein. The absence of a genetic lesion is indicative of a potential for hair growth.

In another preferred embodiment, the method includes evaluating in a sample of epidermal cells for the expression levels of Wnt to determine overexpression. Overexpression of Wnt is indicative of a potential for hair growth.

In a preferred embodiment, the genetic lesions is evaluated by contacting the sample with a nucleic acid probe capable of hybridizing to Wnt mRNA, e.g., a labeled probe. In another preferred embodiment, expression of Wnt is evaluated with an antibody capable of binding to Wnt protein, e.g., a labeled antibody.

In another aspect, the invention features a method for identifying a compound capable of promoting hair growth. The method includes: contacting a cell capable of expressing a Wnt polypeptide with a test compound; and determining the level of Wnt polypeptide or nucleic acid expression, wherein a compound capable of increasing Wnt polypeptide or nucleic acid expression is indicative of a compound capable of promoting hair growth.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, the compound is a Wnt fragment or analog.

In a preferred embodiment, the method further includes evaluating a control cell, e.g., an identical cell which is not treated with the compound.

In a preferred embodiment, the cell is: an epidermal cell, e.g., an epidermal cell from a hair follicle; a DP cell.

In a preferred embodiment, Wnt nucleic acid expression is evaluated using a nucleic acid probe, e.g., a labeled probe, capable of hybridizing to a Wnt nucleic acid molecule, e.g., Wnt mRNA. In a preferred embodiment, Wnt nucleic acid expression, e.g., DNA expression, is evaluated by contacting a compound with a Wnt nucleic acid molecule, e.g., a regulatory sequence of a Wnt nucleic acid molecule, and evaluating Wnt transcription, *in vitro* or *in vivo*, e.g., Wnt transcription is evaluated by determining a cell activity, e.g., using a marker gene, e.g., a lacZ gene or green fluorescence protein (GFP) gene, fused to the regulatory sequence of Wnt and following production of the marker.

In a preferred embodiment, Wnt polypeptide expression is evaluated using an anti-Wnt antibody, e.g., a labeled anti-Wnt antibody.

In another aspect, the invention features a method for identifying a compound capable of inhibiting hair growth. The method includes: contacting a cell capable of expressing a

Wnt polypeptide with a test compound; and determining the level of Wnt polypeptide or nucleic acid expression in the presence and absence of the compound, wherein a compound capable of decreasing Wnt polypeptide or nucleic acid expression is indicative of a compound capable of inhibiting hair growth.

In a preferred embodiment, the Wnt polypeptide is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, Wnt nucleic acid expression is evaluated using a nucleic acid probe, e.g., a labeled probe, capable of hybridizing to a Wnt nucleic acid molecule, e.g., Wnt mRNA. In preferred embodiment, Wnt nucleic acid expression, e.g., DNA expression, is evaluated by contacting a compound with a Wnt nucleic acid molecule, e.g., a regulatory sequence of a Wnt nucleic acid molecule, and evaluating Wnt transcription, *in vitro* or *in vivo*, Wnt transcription is evaluated by determining a cell activity, e.g., using a marker gene, e.g., a lacZ gene or a GFP gene, fused to the regulatory sequence of Wnt and following production of the marker.

In a preferred embodiment, Wnt polypeptide expression is evaluated using an anti-Wnt antibody, e.g., a labeled anti-Wnt antibody.

In another aspect, the invention features a method of culturing a DP cell. For example, a human or non-human, e.g., rodent, e.g., rat or mouse, DP cell. The method includes culturing the DP cell in the presence of an increased level of Wnt, another protein involved in activating the Wnt-β-catenin signaling pathway, e.g., β-catenin and/or LEF-1, and/or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin.

In a preferred embodiment, the level of Wnt is increased over DP cells in the absence of Wnt.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, the DP cell is propagated *in vitro*. In a preferred embodiment, the DP cell is cultured to increase the number of DP cells.

In a preferred embodiment, a Wnt polypeptide or a functional fragment or analog thereof is added to the culture. In another preferred embodiment, an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, is added to the culture. Examples of agents which can mimic an effect of Wnt promoted signal transduction include inhibitors of GSK3β kinase such as lithium chloride or similar small ions.

In another preferred embodiment, the DP cell is cultured in the presence of a cell which expresses a Wnt polypeptide or a functional fragment or analog thereof.

In a preferred embodiment, the DP cell is obtained from a subject, cultured with an increased level of Wnt, or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, and then returned to the same or a different subject.

In a preferred embodiment, the DP cell is maintained in culture and then the cultured DP cells are returned to the same or a different subject to increase the amount of hair growth in the individual.

In another preferred embodiment, the invention features a method of providing and maintaining a dermal papilla cell graft, e.g., a DP graft for hair transplantation procedures. The method includes culturing a DP cell or DP cells in the presence of Wnt or a fragment or analog thereof, another protein involved in activating the Wnt-β-catenin signaling pathway (e.g., β-catenin and/or LEF-1) or a fragment or analog thereof, and/or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin.

In a preferred embodiment, the DP cell is propagated *in vitro*. In a preferred embodiment, the DP cell is propagated *in vitro* to increase the number of DP cells.

In a preferred embodiment, a Wnt polypeptide or a functional fragment or analog thereof is added to the culture. In another preferred embodiment, an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, is added to the culture.

Examples of agents which mimic an effect of Wnt promoted signal transduction include inhibitors of GSK3β kinase such as lithium chloride or similar small ions.

In another preferred embodiment, the DP cell is cultured in the presence of a cell which expresses a Wnt polypeptide or a functional fragment or analog thereof.

In a preferred embodiment, the DP cell is obtained from a subject, cultured with an increased level of Wnt, or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, and then returned to the same or a different subject.

In another aspect, the invention features a media for culturing DP cells which includes a Wnt polypeptide or a functional fragment or analog thereof, or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. Examples of agents which mimic an effect of Wnt promoted signal transduction include inhibitors of GSK3β kinase such as lithium chloride or similar small ions.

In another aspect, the invention features a method of promoting or maintaining anagen phase gene expression of DP cells. The method includes increasing the level of Wnt protein or mimicking an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, to thereby promote or maintain anagen phase gene expression in the DP cells. In another preferred embodiment, the method includes increasing activation of the Wnt-β-catenin signaling pathway, to thereby promote or maintain anagen phase gene expression in DP cells.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, Wnt level is increased or an effect of Wnt promoted signal transduction is mimicked by administering an agent which increases the level of Wnt protein production and/or which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. An agent which increases the level of Wnt protein and/or mimics an effect of

Wnt promoted signal transduction can be one or more of: a Wnt polypeptide or a functional fragment or analog thereof, as described herein; a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof, as described herein; an agent which increases Wnt nucleic acid expression, e.g., a small molecule which binds to the promoter region of Wnt; an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. Examples of agents which can mimic Wnt promoted signal transduction include inhibitors of GSK3β, e.g., lithium chloride or similar small ions, agents which bind Frizzled and mimic Wnt binding, e.g., anti-Frizzled antibodies, or other naturally or non-naturally occurring Frizzled binding ligands.

In a preferred embodiment, the method can be performed *in vitro* or *in vivo*. For example, the DP cells can be maintained in anagen phase in culture, and then administered to a subject, e.g., to increase hair growth. Such methods can include maintaining DP cells in culture in the presence of Wnt or an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. In one embodiment, Wnt and/or an agent which mimics an effect of Wnt promoted signal transduction, e.g., an inhibitor of GSK3β kinase, e.g., lithium chloride or similar small ions, can be added to the culture. In another embodiment, the DP cell can be co-cultured with a cell which expresses Wnt, e.g., a cell which naturally expressed Wnt or has been genetically engineered to express Wnt. DP cells maintained in anagen phase can then be used, e.g., in DP graft procedures. The DP cells can be obtained from the subject who will be receiving the DP graft (i.e., autologous cells), or can be obtained from a different subject (e.g., allogeneic or xenogeneic cells).

In a preferred embodiment, Wnt is increased by administering, e.g., introducing, a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof, into a particular cell, e.g., an epidermal cell or a DP cell, and/or into a subject. The nucleotide sequence can be a genomic sequence or a cDNA sequence. The nucleotide sequence can include: a Wnt coding region; a promoter sequence, e.g., a promoter sequence from a Wnt gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR from a Wnt gene or from another gene, a 3' UTR, e.g., a 3' UTR from a Wnt gene or from another gene; a polyadenylation site; an insulator sequence.

In another preferred embodiment, the level of Wnt protein is increased by increasing the level of expression of an endogenous Wnt gene, e.g., by increasing transcription of the Wnt gene. In a preferred embodiment, transcription of the Wnt gene is increased by: altering the regulatory sequences of the endogenous Wnt gene, e.g., by the addition of a positive regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); the deletion of a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and/or replacement of the endogenous regulatory sequence, or elements therein, with that of another gene, thereby allowing the coding region of the Wnt gene to be transcribed more efficiently.

In another preferred embodiment, the method can include introducing a cell, e.g., a cell which expresses and preferably secretes a Wnt protein, into a subject. In a preferred embodiment, the cell has been genetically modified to express a Wnt protein, or a fragment or an analog thereof. The cell can be an autologous, allogeneic, or xenogeneic cell, but is preferably autologous. The cell can be any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell, an endothelial cell. Preferably the cell is an epithelial cell, e.g., an epidermal cell or a DP cell. The cell can be introduced into a subject to increase the level of Wnt protein.

In a preferred embodiment, the agent which increases the level of Wnt protein and/or mimics an effect of Wnt promoted signal transduction is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered: by continuous administration, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt protein level is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, anagen phase gene expression is promoted or maintained in: the subject's scalp; the subject's face, e.g., upper lip and/or chin.

In another aspect, the invention features a method of promoting or maintaining hair inductive activity. The method includes increasing the level of Wnt protein and/or mimicking an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin, to thereby promote or maintain hair inductive activity.

In a preferred embodiment, the Wnt protein is: Wnt3, e.g., Wnt3a or Wnt 3b; Wnt 4; Wnt 7, e.g., Wnt 7a or 7b. In a particularly preferred embodiment, the Wnt protein is Wnt3, most preferably Wnt3a.

In a preferred embodiment, Wnt is increased by administering an agent which increases the level of Wnt protein production and/or activity. An agent which increases the level of Wnt protein can be one or more of: a Wnt polypeptide or a functional fragment or analog thereof; a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof; an agent which increases Wnt nucleic acid expression, e.g., a small molecule which binds to the promoter region of Wnt; an agent which mimics an effect of Wnt promoted signal transduction, e.g., inhibition of β-catenin phosphorylation, e.g., by inhibition of GSK3β kinase, or accumulation of β-catenin. Examples of agents which mimic an effect of Wnt promoted signal transduction include inhibitors of GSK3β kinase such as lithium chloride or similar small ions

In a preferred embodiment, Wnt is increased by administering, e.g., introducing, a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof, into a particular cell, e.g., an epidermal cell or a DP cell, in the subject. The nucleotide sequence can be a genomic sequence or a cDNA sequence. The nucleotide sequence can include: a Wnt coding region; a promoter sequence, e.g., a promoter sequence from a Wnt gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR from a Wnt gene or from another gene, a 3' UTR, e.g., a 3' UTR from a Wnt gene or from another gene; a polyadenylation site; an insulator sequence.

In another preferred embodiment, the level of Wnt protein is increased by increasing the level of expression of an endogenous Wnt gene, e.g., by increasing transcription of the Wnt gene. In a preferred embodiment, transcription of the Wnt gene is increased by: altering the regulatory sequences of the endogenous Wnt gene, e.g., by the addition of a positive

regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); the deletion of a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and/or replacement of the endogenous regulatory sequence, or elements therein, with that of another gene, thereby allowing the coding region of the Wnt gene to be transcribed more efficiently.

In another preferred embodiment, the method can include introducing a cell, e.g., a cell which expresses and preferably secretes a Wnt protein, into a subject. In a preferred embodiment, the cell has been genetically modified to express a Wnt protein, or a fragment or an analog thereof. The cell can be an autologous, allogeneic, or xenogeneic cell, but is preferably autologous. The cell can be any cell type, e.g., a fibroblast, a keratinocyte, an epithelial cell, an endothelial cell. Preferably the cell is an epithelial cell, e.g., an epidermal cell or a DP cell. The cell can be introduced into a subject to increase the level of Wnt protein and/or to mimic an effect of Wnt promoted signal transduction.

In a preferred embodiment, the agent which increases the level of Wnt protein and/or mimics Wnt promoted signal transduction is administered, e.g., by topically administering the agent; systemically administering the agent; orally administering the agent; or injecting the agent, preferably dermally or subcutaneously. In preferred embodiments, the compound is administered using a suitable delivery vehicle, for example, a surfactant or an agent which increases permeability in the skin, e.g., an SDS or DMSO containing formulation. Preferably, the agent is included in a composition for topical use, e.g., the composition is a gel, cream, or liquid. In a preferred embodiment, the agent is administered: by continuous administration, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt protein level and/or the Wnt signaling pathway is maintained for a selected period, e.g., 10, 20, 30, 50, 90, 180, 365 days or more. In another preferred embodiment, administration of the agent is repeated, e.g., is repeated at least 1, 2, 3, 5, 10, 20 or more times.

In a preferred embodiment, hair inductive activity is promoted or maintained on: the subject's scalp; the subject's face, e.g., upperlip and/or chin.

A "treatment", as used herein, includes any therapeutic treatment, e.g., the administration of a therapeutic agent or substance, e.g., a drug.

The term "increasing hair growth" as used herein refers to increasing the number or density or distribution of follicles or hair shafts or otherwise increasing the growth of hair.

As used herein, the term "subject" refers an animal, e.g., a mammal, e.g., a human. The mammal can be a human or non-human mammal, e.g., a swine, a bird, a cat, a dog, a monkey, a goat, or a rodent, e.g., a rat or a mouse. The animal can be a transgenic animal, e.g., a transgenic rodent, e.g., a transgenic rat or mouse.

"Regulatory sequence" refers to any or all of the DNA sequences that controls gene expression. An example of a regulatory sequence includes: a promoter, a positive regulatory element (such as an enhancer or a DNA-binding site for a transcriptional activator); a negative regulatory element (such as a DNA-binding site for a transcriptional repressor) and an insulator.

"Heterologous" refers to DNA or tissue which is derived from a different species.

"Heterologous regulatory sequence" refers to a sequence which is not the normal regulatory sequence of that gene

The terms "peptides", "proteins", and "polypeptides" are used interchangeably herein.

The term "small molecule", as used herein, includes peptides, peptidomimetics, or non-peptidic compounds, such as organic molecules, having a molecular weight less than 2,000, preferably less than 1,000.

The term "effects of Wnt-promoted signal transduction" refers to one or more of the biochemical effects (e.g., modulation of e.g., protein binding interactions, phosphorylation or transcription) in a cell, e.g., a DP cell, initiated by Wnt signaling, e.g., by Wnt binding to Frizzled. Effects of Wnt promoted signal transduction can include Wnt binding to Frizzled; inhibition of GSK3β mediated phosphorylation; inhibition of phosphorylation-dependent degradation of β-catenin; accumulation of β-catenin protein in the cytoplasm; stabilization of cellular β-catenin; β-catenin accumulation in the cytoplasm; β-catenin binding to Lef1; translocation of the β- catenin-Lef1 complex to the nucleus; and stimulation of transcription from associated genes. Components of Wnt-promoted signal transduction can include Frizzled protein, e.g., Frizzled-7 (frz-7), disheveled proteins, e.g., disheveled-2 (dsh-2), GSK3, beta catenin, Lef1, and Lef/TFC. Other effects and components of the Wnt signaling pathway are described in Arias et al. (1999) *Curr. Opin. Genet. & Dev.* 9:447-454, which is incorporated herein by reference.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Detailed Description of the Invention

The present invention is based, in part, on the discovery that Wnt proteins expressed in the follicular epithelium maintain anagen phase gene expression in dermal papilla cells and that hair inductive activity is also maintained by Wnt signaling.

Both formation of the hair follicle during embryonic development and the cyclical growth, quiescence and regeneration of the hair shaft during the hair cycle are dependent on reciprocal signaling between the epidermal and dermal components of the follicle.

In the Wnt signaling pathway, Wnt, which is a soluble molecule, binds Frizzled (Frz), a cell surface receptor, found on various types of cells. In the presence of disshelved, binding of Wnt to Frz results in the inhibition of GSK3β mediated phosphorylation and subsequent phosphorylation-dependent degradation of β-catenin. Thus, Wnt binding stabilizes cellular β-catenin. In the presence of Wnt binding, β-catenin accumulates in the cytoplasm and binds to Lef1. The β-catenin-Lef1 complex then translocates to the nucleus, where it mediates transcriptional activation.

Production of a Transgenic Mouse Expressing Green Fluorescent Protein in DP Cells

A transgenic mouse line that specifically expresses green fluorescent protein (GFP) in DP cells during the anagen (growth) phase of the hair cycle was used to purify GFP expressing DP cells and study the signals required to maintain GFP expression. The transgenic mouse line was generated as described in Kishimoto et al. (1999) *Proc. Natl Acad. Sci USA* 96:7336-7341. It was found that isolated transgenic anagen hair follicles show GFP fluorescence in the DP. The versican-GFP transgene is active during anagen but is shut off during catagen and telogen of hair growth. Therefore, transgene expression was correlated with the presumed profile of inductive activity in the DP.

RT-PCR Analysis

Total RNA was isolated and RT PCR performed as described in Kishimoto et al. (1999) *Proc. Natl Acad. Sci. USA* 96:7336-7341, using an annealing temperature of 58°C. The primers employed are listed below. With the exception of Wnts 4, 5 and 7, the primers were designed to distinguish between mouse and chicken orthologues and the lack of cross reaction to chick sequences was confirmed on cDNA from feeder cells alone.

Wnt3: G CGC CCT GGC TCA CTA C (SEQ ID NO:1)
and ATG CTG CTG CTG CTG GCC (SEQ ID NO:2) for 30 cycles
Wnt4: TGA TCC AGA GGC AGG TGC AG (SEQ ID NO:3)
and CTT CTC CAG TTC TCC ACT GC (SEQ ID NO:4) for 36 cycles
Wnt5a: CTG TTG AC TGC ACC AGC TT (SEQ ID NO:5) and
TCA AGG AAT GCC AGT ACC AGT ACC AG (SEQ ID NO:6) for 30 cycles
Frizzled-7: CTG CTA GAG GAC CGT GCC (SEQ ID NO:7)
and AGG TGC GTT CCC AGT GCT (SEQ ID NO:8) for 36cycles
Disheveled-2: CAT CCT TCA GCA GTG TCA (SEQ ID NO:9)
and CGT CAT TGT CAT TCA GAG (SEQ ID NO:10) for 36 cycles
GSK-3β: CAG GGC ACC AGA GTT GAT (SEQ ID NO:11)
and GCA GAA GCG GCG TTA TTG (SEQ ID NO:12) for 30 cycles
β-catenin: CCA CCA GCT AGG CGC ACT (SEQ ID NO:13)
and GGG CTC AGA GGG TCC GAG (SEQ ID NO:14) for 30 cycles
LEF-1: ACT GTC AGG CGA CAC TTC C (SEQ ID NO:15)
and TGC ACG TTG GGA AGG AGC (SEQ ID NO:16) for 36 cycles
Shh: for 30 cycles (SEQ ID NO:17 & 18)
Patched-1: AGC CTC ACA GTA ACA CCC (SEQ ID NO: 20)
and TGT TCT CCT CCA GCA TGA (SEQ ID NO:21) for 36 cycles
Gli-1: TTG GGG ATG CTG GAT GGG (SEQ ID NO:22)
and CGG TCA CTG GCA TTG CTA (SEQ ID NO:23) for 36 cycles
β-actin: 5'-CCA CAC CCG CCA CCA GTT C-3' (SEQ ID NO:24)
and 5'-GAGGAAGAGGATGCGGCA-3' (SEQ ID NO:25) for 26 cycles
GFP: 5'-TGCAGTGCTTCAGCCGCTAC- 3' (SEQ ID NO: 26) AND
5'-CTCGTTGGGGTCTTTGCTCA-3' (SEQ ID NO: 27) for 26 cycles.

Cell Culture

Fresh mouse pelage DP cells were obtained from versican GFP transgenic newborn skin by high-speed cell sorting (MoFLO) as described previously in Kishimoto et al., *supra*. Chick embryo fibroblasts (CEFs) were infected with RCASBP(A) retroviral vectors encoding chick Shh, as described in Riddle et al. (1993) *Cell* 75:1401-1416, Wnt3a, as described in Kengaku et al. (1998) *Science* 280:1274-1277, Wnt4, Wnt5a (Noramly and Morgan, in prep), or Wnt7a, as described in Riddle et al. (1995) *Cell* 83:631-640, or vector alone as described, as described in Morgan et al. in *Methods in Cell Biology* (ed. Bonner-Fraser) pp.185-218 (Academic Press, San Diego, 1996). GFP positive mouse DP cells were added onto 30-50% confluent feeder cells to achieve a ratio of 1:3 (mouse:chick). For the flow cytometric analysis, cells were co-cultured in 24 well dishes for 48-96 hrs. For the grafting experiment co-cultured cells were passaged 2-3 times in 10 cm dishes to generate the $2x10^6$ cells required for each graft.

Flow Cytometry

Cells were trypsinized and resuspended in 0.5 ml PBS with 1% BSA. Flow cytometric analysis was performed by FACScan (Becton Dickinson). DP cells were labeled with mouse MHC class II monoclonal antibody and incubated with secondary IgG conjugated to PE. The PE negative avian cells fell below the lower gate in Fig. 3 and were excluded from the analysis of GFP expression.

Reconstitution Assay

The reconstitution assay was performed as described in Kishimoto et al., *supra*. Primary keratinocytes were prepared from 2 newborn pups per graft and combined with $2x10^6$ DP cells in the graft chamber. Hair growth was monitored two weeks after grafting and weekly thereafter.

Production of Cell Culture of GFP Expressing DP Cells

Anagen dermal papilla cells from the skin of the transgenic mouse described above were purified to homogeneity using a fluorescence activated cell sorter and were shown to retain hair inductive activity in a skin reconstitution assay. However, using flow cytometric analysis of GFP expression in DP cells immediately after isolation and after 90 hours in culture, it was found that the inductive activity and GFP expression of the DP cells were rapidly lost in culture. This suggests that a factor normally supplied by epidermal cells was required to maintain DP cells in the anagen state.

Analysis of the Role of Shh in Anagen Phase

One candidate for the signal supplied by the epidermal cells was Shh, which is expressed in the epidermal component of the hair follicle and is required for the maturation of the dermal papilla during embryonic development. See, e.g., St. Jacques (1998) *Curr. Biol.* 8:1058-1068. During the hair cycle, exogenous Shh can accelerate the transition from telogen to anagen. Sato et al. (1999) *J. Clin. Invest.* 104:855-864. Expression of Shh, patched-1 (ptc-1), Gli-1 and the control of β-actin genes was analyzed in DP cells immediately after isolation and after three passages in culture. In skin from newborn transgenic mice undergoing active hair growth, Shh mRNA was detected in the GFP negative population of sorted cells, which includes follicular epidermis, and was absent from the dermal papilla cells. Ptc-1 and Gli-1 are expressed in isolated DP but transcription levels decreased upon passage in culture. Transcriptional feedback in the Shh signal transduction cascade results in increased accumulation of mRNA encoding two of its components, patched (ptc) and Gli-1, in response to Shh signaling. This induction serves as an indication of response to the Shh signal. Goodrich et al. (1996) *Genes Dev.* 10:301-312; Marigo et al. (1996) *Development* 122:1225-1233. Message from both genes was readily detected in freshly sorted DP cells, but the abundance of both messages decreases to undetectable levels when isolated DP cells were cultured in the absence of follicular epithelium. Thus, Shh signaling from follicular epithelium to the dermal papilla occurs in the hair follicle and could cause DP activation in anagen. Therefore, it was evaluated whether Shh signaling was sufficient to rescue either GFP gene expression or hair inductive activity in DP cells

maintained in culture. Freshly isolated DP cells were co-cultured with CEFs expressing Shh or infected with a control vector. The use of heterospecific feeder cells allowed analyzation of gene expression in the DP cells by PCR with species-specific primers and confirms that the DP cells received the Shh signal as demonstrated by induction and maintenance of both ptc-1 and Gli-1, while expression of both genes is decreased in DP cells co-cultured with control feeders. β-actin sequences were used to normalize input cDNA and murine specific primers were employed to discriminate between gene expression in the DP and feeder cell populations. However, as demonstrated by flow cytometric analysis of DP cells co-cultures with feeder layers producing Wnt3 or Shh or infected with control vectors, GFP expression declined at identical rates in Shh treated and control populations. In addition, the ratio of GFP positive and GFP negative cells was confirmed for each treatment. To confirm the correlation between GFP expression and maintenance of the anagen state, these cells were mixed with keratinocytes and grafted in bubble chambers on the backs of nude mouse hosts. In this assay, the hairless skin which forms in the absence of active DP cells was observed whether or not the DP cells had been cultured in the presence of Shh. Both grafts of control or Shh treated DP cells showed only occasional hairs three weeks after grafting to the nude mouse model. See Table 1.

As *in vivo* experiments have suggested that Shh stimulates the transition from telogen to anagen, it was also assessed whether Shh signaling was sufficient to activate isolated DP cells which had been maintained in culture until they were formally analogous to telogen DP cells in that both GFP expression and hair inductive activity were lost. Again, no change in either property was observed. Thus, although Shh may initiate anagen *in vivo*, it is likely that it acts on the DP in part indirectly, possibly by induction of a secondary signal in the epidermis.

Analysis of the Role of Wnt in Anagen Phase

A search of the sequence in the versican enhancer used to drive GFP expression in the DP revealed a Lef/TCF binding motif and suggested that a Wnt might serve as the signal from the epidermis which activates the DP cells. This binding site appears to be required for GFP expression because no GFP expression was observed in ten independent transgenic lines when this region was deleted from the expression construct. However, deletions from

elsewhere in the construct had no effect on GFP expression in transgenic mice. The Lef/TCF family of DNA binding proteins mediates the transcriptional effects of Wnt signaling through the β-catenin pathway. Arias et al. (1999) *Curr. Opin. Genet. & Dev.* 9:447-454. Wnts are secreted glycoproteins which bind to Frizzled. In a process dependent on disheveled proteins, Frizzled receptor engagement inhibits phosphorylation of β catenin by a complex including GSK3. This results in accumulation of β-catenin protein in the cytoplasm and translocation to the nucleus where it can bind Lef/TCF and stimulate transcription from associated genes. In freshly isolated DP cells, Frizzled-7 (frz-7), disheveled-2 (dsh-2), GSK3, β catenin, Lef1 and the GFP transgene are all expressed. Thus, the components of this signal transduction cascade were expressed in freshly isolated DP cells. After three passages, expression of frz-7, dsv-2 and Lef1 are reduced. Furthermore, Wnt3a is expressed in the follicular matrix cells and Wnt3a, 5a and 7a transcripts were detected in the GFP negative population from dissociated skin which includes the follicular epithelia from anagen hair follicles. Thus, all of these Wnts are candidates to mediate signaling to the dermal papilla.

Feeder cells expressing Wnts 3a, 4, 5a or 7a were used to test the effects of Wnt signaling on freshly isolated DP cells. Co-culture with Wnts3a or 7a resulted in maintenance of GFP fluorescence in the majority of the DP cells as demonstrated by FACS analysis. RT PCR confirmed that this reflects increased levels of GFP RNA rather than stabilization of the encoded protein. In particular, GFP RNA levels are maintained by exposure to Wnt3a but not Shh or control feeder layers. Wnt 4 expressing cells also showed some maintenance of GFP expression but were less potent than either Wnt3a or 7a, while Wnt5a had no effect on DP GFP expression. As suggested by GFP expression, the hair inductive activity of DP cells was also maintained in culture by Wnt signaling. When the murine DP cells were resorted, combined with keratinocytes and used to reconstitute skin on a nude mouse host, Wnt3a or 7a treated cells showed a dramatic increase in hair growth compared to cells co-cultured with control feeders or Shh expression cells. The control and Shh treated DP cell grafts only showed an occasional hair three weeks after grafting, whereas DP cells exposed to Wnt3a formed a dense patch of hair in the graft. See Table 1.

While Wnt3a is sufficient to maintain DP cells in the anagen state, it cannot reactivate GFP expression or hair inductive activity in cells which have lost these properties in culture

(data not shown). Analysis of transcripts encoding components of the Wnt signal transduction cascade reveals that Lef-1, disheveled-2 and Frizzled-7 are all downregulated after maintenance in culture in the absence of Wnts and this could explain the failure of exogenous Wnt to reactivate GFP expression in these cells. Maintenance of expression of proteins required for Wnt signal transduction by other factors expressed locally in the epidermis could contribute to the coordination of morphogenesis in the follicle. The findings reported here suggest the possibility that a single Wnt expressed in the epidermis could coordinate development in both the dermis and epidermis.

The results demonstrate that Wnt3a, and possibly other Wnts, expressed in the anagen hair follicle can act as inductive signals to maintain the dermal papilla in an anagen state. The results also suggest that Shh signaling is not sufficient to initiate or maintain the anagen state of DP cells, and may therefore act at least in part indirectly to promote the transition to anagen in vivo. Finally these results extend the correlation between GFP expression in these transgenic DP cells and their ability to induce hair growth and thus suggest further use of this approach to dissect signaling between epithelia and mesenchyme to coordinate morphogenesis.

Wnt Polypeptides and Nucleic Acid Sequences Encoding Wnt

Wnt polypeptides can be obtained in several ways including isolation of Wnt or expression of a sequence encoding Wnt by genetic engineering methods. The nucleotide sequences of various Wnt proteins from various species are known. *See, e.g.*, Gavin et al. (1990) *Genes Dev.* 4:2319-2332; Lee et al. (1995) *Proc. Natl Acad. Sci USA* 92:2268-2272; and, Christiansen et al. (1995) *Mech. Dev.* 51:341-350 (describing, e.g., murine Wnt1, Wnt2, Wnt3a, Wnt3b, Wnt4, Wnt5a, Wnt 5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt10b, Wnt11, Wnt12) and Vant Veer et al. (1984) Mol. Cell Biol. 4:2532-2534; Wainwright et al. (1988) EMBO J. 7:1743-1748; and, PCT Publication WO 95/17416 (describing, e.g., human Wnt1, Wnt2, Wnt3, Wnt4, Wnt5a, Wnt7a and Wnt7b).

Analogs of Wnt or Other Proteins Involved in the Wnt-β-catenin Signaling Pathway

Analogs can differ from naturally occurring protein in amino acid sequence or in ways that do not involve sequence, or both. Non-sequence modifications include *in vivo* or

in vitro chemical derivatization of the protein. Non-sequence modifications include changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation.

Preferred analogs include a protein, e.g., Wnt, (or biologically active fragments thereof) whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish the biological activity. In a preferred embodiment, the sequence can differ from wild-type sequence by 1, 2, 3, 5, 10, but not more than 20 to 30 amino acid residues. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other conservative substitutions can be taken from the table below.

TABLE 1

CONSERVATIVE AMINO ACID REPLACEMENTS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D- homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β-Ala Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met,D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met

Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4- carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Other analogs within the invention are those with modifications which increase peptide stability; such analogs may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Production of Fragments and Analogs

Generation of Fragments

Fragments of a protein can be produced in several ways, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNA's which encode an array of fragments. DNA's which encode fragments of a protein can also be generated by random shearing, restriction digestion or a combination of the above-discussed methods.

Fragments can also be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Random Methods

Amino acid sequence variants of a protein can be prepared by random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein. Useful methods include PCR mutagenesis and saturation mutagenesis. A library of random amino acid sequence variants can also be generated by the synthesis of a set of degenerate oligonucleotide sequences. (Methods for screening proteins in a library of variants are elsewhere herein.)

PCR Mutagenesis

In PCR mutagenesis, reduced Taq polymerase fidelity is used to introduce random mutations into a cloned fragment of DNA (Leung et al., 1989, *Technique* 1:11-15). This is a very powerful and relatively rapid method of introducing random mutations. The DNA region to be mutagenized is amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, e.g., by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments are inserted into appropriate cloning vectors to provide random mutant libraries.

Saturation Mutagenesis

Saturation mutagenesis allows for the rapid introduction of a large number of single base substitutions into cloned DNA fragments (Mayers et al., 1985, *Science* 229:242). This technique includes generation of mutations, e.g., by chemical treatment or irradiation of single-stranded DNA *in vitro*, and synthesis of a complimentary DNA strand. The mutation frequency can be modulated by modulating the severity of the treatment, and essentially all possible base substitutions can be obtained. Because this procedure does not involve a

genetic selection for mutant fragments both neutral substitutions, as well as those that alter function, are obtained. The distribution of point mutations is not biased toward conserved sequence elements.

Degenerate Oligonucleotides

A library of homologs can also be generated from a set of degenerate oligonucleotide sequences. Chemical synthesis of a degenerate sequences can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The synthesis of degenerate oligonucleotides is known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

Generation of Analogs: Production of Altered DNA and Peptide Sequences by Directed Mutagenesis

Non-random or directed, mutagenesis techniques can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting the target residue, or (3) inserting residues of the same or a different class adjacent to the located site, or combinations of options 1-3.

Alanine Scanning Mutagenesis

Alanine scanning mutagenesis is a useful method for identification of certain residues or regions of the desired protein that are preferred locations or domains for mutagenesis, Cunningham and Wells (*Science* 244:1081-1085, 1989). In alanine scanning, a residue or

group of target residues are identified (e.g., charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid (most preferably alanine or polyalanine). Replacement of an amino acid can affect the interaction of the amino acids with the surrounding aqueous environment in or outside the cell. Those domains demonstrating functional sensitivity to the substitutions are then refined by introducing further or other variants at or for the sites of substitution. Thus, while the site for introducing an amino acid sequence variation is predetermined, the nature of the mutation per se need not be predetermined. For example, to optimize the performance of a mutation at a given site, alanine scanning or random mutagenesis may be conducted at the target codon or region and the expressed desired protein subunit variants are screened for the optimal combination of desired activity.

Oligonucleotide-Mediated Mutagenesis

Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, e.g., Adelman et al., (*DNA* 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to a DNA template, where the template is the single-stranded form of a plasmid or bacteriophage containing the unaltered or native DNA sequence of the desired protein. After hybridization, a DNA polymerase is used to synthesize an entire second complementary strand of the template that will thus incorporate the oligonucleotide primer, and will code for the selected alteration in the desired protein DNA. Generally, oligonucleotides of at least 25 nucleotides in length are used. An optimal oligonucleotide will have 12 to 15 nucleotides that are completely complementary to the template on either side of the nucleotide(s) coding for the mutation. This ensures that the oligonucleotide will hybridize properly to the single-stranded DNA template molecule. The oligonucleotides are readily synthesized using techniques known in the art such as that described by Crea et al. (*Proc. Natl. Acad. Sci.* USA, 75: 5765[1978]).

Cassette Mutagenesis

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells et al. (*Gene*, 34:315[1985]). The starting material is a plasmid

(or other vector) which includes the protein subunit DNA to be mutated. The codon(s) in the protein subunit DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the desired protein subunit DNA. After the restriction sites have been introduced into the plasmid, the plasmid is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures. The two strands are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 3' and 5' ends that are comparable with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated desired protein subunit DNA sequence.

Combinatorial Mutagenesis

Combinatorial mutagenesis can also be used to generate mutants. For example, the amino acid sequences for a group of homologs or other related proteins are aligned, preferably to promote the highest homology possible. All of the amino acids which appear at a given position of the aligned sequences can be selected to create a degenerate set of combinatorial sequences. The variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential sequences are expressible as individual peptides, or alternatively, as a set of larger fusion proteins containing the set of degenerate sequences.

Primary High-Through-Put Methods for Screening Libraries of Peptide Fragments or Homologs

Various techniques are known in the art for screening generated mutant gene products. Techniques for screening large gene libraries often include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of

vectors, and expressing the genes under conditions in which detection of a desired activity, assembly into a trimeric molecules, binding to natural ligands, e.g., a Frz receptor or substrates, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, e.g., by random mutagenesis techniques.

Two Hybrid Systems

Two hybrid (interaction trap) assays can be used to identify a protein that interacts with Wnt. These may include agonists, superagonists, and antagonists. (The subject protein and a protein it interacts with are used as the bait protein and fish proteins.). These assays rely on detecting the reconstitution of a functional transcriptional activator mediated by protein-protein interactions with a bait protein. In particular, these assays make use of chimeric genes which express hybrid proteins. The first hybrid comprises a DNA-binding domain fused to the bait protein. e.g., a Wnt molecule or a fragment thereof. The second hybrid protein contains a transcriptional activation domain fused to a "fish" protein, e.g. an expression library. If the fish and bait proteins are able to interact, they bring into close proximity the DNA-binding and transcriptional activator domains. This proximity is sufficient to cause transcription of a reporter gene which is operably linked to a transcriptional regulatory site which is recognized by the DNA binding domain, and expression of the marker gene can be detected and used to score for the interaction of the bait protein with another protein.

Display Libraries

In one approach to screening assays, the candidate peptides are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to bind an appropriate receptor protein via the displayed product is detected in a "panning assay". For example, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, a detectably labeled ligand can be used to score for

potentially functional peptide homologs. Fluorescently labeled ligands, e.g., receptors, can be used to detect homolog which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, to be separated by a fluorescence-activated cell sorter.

A gene library can be expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations well over 10^{13} phage per milliliter, a large number of phage can be screened at one time. Second, since each infectious phage displays a gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd., and f1 are most often used in phage display libraries. Either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle. Foreign epitopes can be expressed at the NH_2-terminal end of pIII and phage bearing such epitopes recovered from a large excess of phage lacking this epitope (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al. (1993) *EMBO J* 12:725-734; Clackson et al. (1991) *Nature* 352:624-628; and Barbas et al. (1992) *PNAS* 89:4457-4461).

A common approach uses the maltose receptor of *E. coli* (the outer membrane protein, LamB) as a peptide fusion partner (Charbit et al. (1986) *EMBO* 5, 3029-3037). Oligonucleotides have been inserted into plasmids encoding the LamB gene to produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, e.g., to antibodies, and can elicit an immune response when the cells are administered to animals. Other cell surface proteins, e.g., OmpA (Schorr et al. (1991) *Vaccines 91*, pp. 387-392), PhoE (Agterberg, et al. (1990) *Gene* 88, 37-45), and PAL (Fuchs et al. (1991) *Bio/Tech* 9, 1369-1372), as well as large bacterial surface structures have served as vehicles for peptide display. Peptides can be fused to pilin, a protein which polymerizes to form the pilus-a conduit for interbacterial exchange of genetic information (Thiry et al. (1989) *Appl. Environ. Microbiol.* 55, 984-993). Because of its role in interacting with other

cells, the pilus provides a useful support for the presentation of peptides to the extracellular environment. Another large surface structure used for peptide display is the bacterial motive organ, the flagellum. Fusion of peptides to the subunit protein flagellin offers a dense array of may peptides copies on the host cells (Kuwajima et al. (1988) *Bio/Tech.* 6, 1080-1083). Surface proteins of other bacterial species have also served as peptide fusion partners. Examples include the *Staphylococcus* protein A and the outer membrane protease IgA of *Neisseria* (Hansson et al. (1992) *J. Bacteriol.* 174, 4239-4245 and Klauser et al. (1990) *EMBO J.* 9, 1991-1999).

In the filamentous phage systems and the LamB system described above, the physical link between the peptide and its encoding DNA occurs by the containment of the DNA within a particle (cell or phage) that carries the peptide on its surface. Capturing the peptide captures the particle and the DNA within. An alternative scheme uses the DNA-binding protein LacI to form a link between peptide and DNA (Cull *et al.* (1992) *PNAS USA* 89:1865-1869). This system uses a plasmid containing the LacI gene with an oligonucleotide cloning site at its 3'-end. Under the controlled induction by arabinose, a LacI-peptide fusion protein is produced. This fusion retains the natural ability of LacI to bind to a short DNA sequence known as LacO operator (LacO). By installing two copies of LacO on the expression plasmid, the LacI-peptide fusion binds tightly to the plasmid that encoded it. Because the plasmids in each cell contain only a single oligonucleotide sequence and each cell expresses only a single peptide sequence, the peptides become specifically and stably associated with the DNA sequence that directed its synthesis. The cells of the library are gently lysed and the peptide-DNA complexes are exposed to a matrix of immobilized receptor to recover the complexes containing active peptides. The associated plasmid DNA is then reintroduced into cells for amplification and DNA sequencing to determine the identity of the peptide ligands. As a demonstration of the practical utility of the method, a large random library of dodecapeptides was made and selected on a monoclonal antibody raised against the opioid peptide dynorphin B. A cohort of peptides was recovered, all related by a consensus sequence corresponding to a six-residue portion of dynorphin B. (Cull et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89-1869)

This scheme, sometimes referred to as peptides-on-plasmids, differs in two important ways from the phage display methods. First, the peptides are attached to the C-terminus of

the fusion protein, resulting in the display of the library members as peptides having free carboxy termini. Both of the filamentous phage coat proteins, pIII and pVIII, are anchored to the phage through their C-termini, and the guest peptides are placed into the outward-extending N-terminal domains. In some designs, the phage-displayed peptides are presented right at the amino terminus of the fusion protein. (Cwirla, et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87, 6378-6382) A second difference is the set of biological biases affecting the population of peptides actually present in the libraries. The LacI fusion molecules are confined to the cytoplasm of the host cells. The phage coat fusions are exposed briefly to the cytoplasm during translation but are rapidly secreted through the inner membrane into the periplasmic compartment, remaining anchored in the membrane by their C-terminal hydrophobic domains, with the N-termini, containing the peptides, protruding into the periplasm while awaiting assembly into phage particles. The peptides in the LacI and phage libraries may differ significantly as a result of their exposure to different proteolytic activities. The phage coat proteins require transport across the inner membrane and signal peptidase processing as a prelude to incorporation into phage. Certain peptides exert a deleterious effect on these processes and are underrepresented in the libraries (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251). These particular biases are not a factor in the LacI display system.

The number of small peptides available in recombinant random libraries is enormous. Libraries of 10^7-10^9 independent clones are routinely prepared. Libraries as large as 10^{11} recombinants have been created, but this size approaches the practical limit for clone libraries. This limitation in library size occurs at the step of transforming the DNA containing randomized segments into the host bacterial cells. To circumvent this limitation, an *in vitro* system based on the display of nascent peptides in polysome complexes has recently been developed. This display library method has the potential of producing libraries 3-6 orders of magnitude larger than the currently available phage/phagemid or plasmid libraries. Furthermore, the construction of the libraries, expression of the peptides, and screening, is done in an entirely cell-free format.

In one application of this method (Gallop et al. (1994) *J. Med. Chem.* 37(9):1233-1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library expressed in an *E. coli* S30 *in vitro* coupled transcription/translation system. Conditions

were chosen to stall the ribosomes on the mRNA, causing the accumulation of a substantial proportion of the RNA in polysomes and yielding complexes containing nascent peptides still linked to their encoding RNA. The polysomes are sufficiently robust to be affinity purified on immobilized receptors in much the same way as the more conventional recombinant peptide display libraries are screened. RNA from the bound complexes is recovered, converted to cDNA, and amplified by PCR to produce a template for the next round of synthesis and screening. The polysome display method can be coupled to the phage display system. Following several rounds of screening, cDNA from the enriched pool of polysomes was cloned into a phagemid vector. This vector serves as both a peptide expression vector, displaying peptides fused to the coat proteins, and as a DNA sequencing vector for peptide identification. By expressing the polysome-derived peptides on phage, one can either continue the affinity selection procedure in this format or assay the peptides on individual clones for binding activity in a phage ELISA, or for binding specificity in a completion phage ELISA (Barret, et al. (1992) *Anal. Biochem* 204,357-364). To identify the sequences of the active peptides one sequences the DNA produced by the phagemid host.

Secondary Screens

The high through-put assays described above can be followed by secondary screens in order to identify further biological activities which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend on the desired activity that needs to be tested. For example, an assay can be developed in which the ability to inhibit an interaction between a protein of interest and its respective ligand can be used to identify antagonists from a group of peptide fragments isolated though one of the primary screens described above.

Therefore, methods for generating fragments and analogs and testing them for activity are known in the art. Once the core sequence of interest is identified, it is routine to perform for one skilled in the art to obtain analogs and fragments.

Peptide Mimetics

The invention also provides for reduction of the protein binding domains of the subject Wnt polypeptides to generate mimetics, e.g. peptide or non-peptide agents. See, for

example, "Peptide inhibitors of human papillomavirus protein binding to retinoblastoma gene protein" European patent applications EP-412,762A and EP-B31,080A.

Non-hydrolyzable peptide analogs of critical residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Fusion Proteins

Polypeptides for modulating the level of Wnt protein can be fused to another protein or portion thereof. For example, a Wnt protein or portion thereof, such as the Frizzled binding portion of Wnt, can be operably linked to another polypeptide moiety to enhance solubility. Examples of a protein which can be fused with Wnt or portions thereof include a plasma protein or fragment thereof, which can improve the circulating half life of Wnt. For example, the fusion protein can be a Wnt-immunoglobulin (Ig) fusion protein in which the Wnt sequence is fused to a sequence derived from the immunoglobulin superfamily. Several soluble fusion protein constructs have been disclosed wherein the extracellular domain of a cell surface glycoprotein is fused with the constant F(c) region of an immunoglobulin. For example, Capon et al. (1989) *Nature* 337(9):525-531, provide guidance on generating a longer lasting CD4 analog by fusing CD4 to an immunoglobulin (IgG1). *See also*, Capon et al., U.S. Patent Numbers: 5,116,964 and 5,428,130 (CD4-IgG fusion constructs); Linsley et al., U.S, Patent Number 5,434,131 (CTLA4-IgG1 and B7-IgG1 fusion constructs); Linsley et al. (1991) *J. Exp. Med.* 174:561-569 (CTLA4-IgG1 fusion constructs); and Linsley et al. (1991) *J. Exp. Med* 173:721-730 (CD28-IgG1 and B7-IgG1 fusion constructs). Such fusion

proteins have proven useful for modulating receptor-ligand interactions and reducing inflammation *in vivo*. For example, fusion proteins in which an extracellular domain of cell surface tumor necrosis factor receptor (TNFR) proteins has been fused to an immunoglobulin constant (Fc) region have been used *in vivo*. *See, for example,* Moreland *et al.* (1997) *N. Engl. J. Med.* 337(3):141-147; and, van der Poll *et al.* (1997) *Blood* 89(10):3727-3734).

Antibodies

The invention also includes antibodies specifically reactive with a subject Wnt polypeptides or Frizzled as well as antibodies specifically reactive with other proteins of the Wnt-β-catenin signaling pathway, e.g., intrabodies. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made as described herein by using standard protocols (See, for example, *Antibodies: A Laboratory Manual* ed. by Harlow and Lane (Cold Spring Harbor Press: 1988)).

A Wnt protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind Wnt using standard techniques for polyclonal and monoclonal antibody preparation. The full-length Wnt protein can be used or, alternatively, antigenic peptide fragments of Wnt can be used as immunogens.

Typically, Wnt or a Wnt peptide is used to prepare antibodies by immunizing a suitable subject, (e.g., rabbit, goat, mouse or other mammal) with the immunogen. An appropriate immunogenic preparation can contain, for example, a recombinant Wnt peptide, or a chemically synthesized Wnt peptide. *See*, e.g., U.S. Patent No. 5,460,959; and co-pending U.S. applications USSN 08/334,797; USSN 08/231,439; USSN 08/334,455; and USSN 08/928,881 which are hereby expressly incorporated by reference in their entirety. The nucleotide and amino acid sequences of Wnt are known and described, for example, in Vant Veer et al. (1984) Mol. Cell. Biol. 4:2532-2534, Gavin et al. (1992) *Gene Dev.* 4:2319-2332; Lee et al. (1995) *Proc. Natl Acad. Sci USA* 92:2268-2272; Christiansen et al. (1995) Mech. Dev. 51:341-350, Wainwright et al. (1988) EMBO J. 7:1743-1748; and, PCT Publication WO 95/17416. The nucleotide and amino acid sequence of other members of the Wnt-β-catenin signal pathway are also known. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant, or similar immunostimulatory

agent. Immunization of a suitable subject with an immunogenic Wnt preparation induces a polyclonal anti-Wnt antibody response.

Anti-Wnt antibodies or fragments thereof can be used to inhibit the levels of Wnt protein. Examples of anti-Wnt antibody fragments include F(v), Fab, Fab' and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of Wnt. A monoclonal antibody composition thus typically displays a single binding affinity for a particular Wnt protein with which it immunoreacts.

Additionally, anti-Wnt antibodies produced by genetic engineering methods, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, can be used. Such chimeric and humanized monoclonal antibodies can be produced by genetic engineering using standard DNA techniques known in the art, for example using methods described in Robinson et al. International Application No. PCT/US86/02269; Akira, et al. European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al. European Patent Application 173,494; Neuberger et al. PCT International Publication No. WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al. European Patent Application 125,023; Better et al., *Science* 240:1041-1043, 1988; Liu et al., *PNAS* 84:3439-3443, 1987; Liu et al., *J. Immunol.* 139:3521-3526, 1987; Sun et al. *PNAS* 84:214-218, 1987; Nishimura et al., *Canc. Res.* 47:999-1005, 1987; Wood et al., *Nature* 314:446-449, 1985; and Shaw et al., *J. Natl. Cancer Inst.* 80:1553-1559, 1988); Morrison, S. L., *Science* 229:1202-1207, 1985; Oi et al., *BioTechniques* 4:214, 1986; Winter U.S. Patent 5,225,539; Jones et al., *Nature* 321:552-525, 1986; Verhoeyan et al., *Science* 239:1534, 1988; and Beidler et al., *J. Immunol.* 141:4053-4060, 1988.

In addition, a human monoclonal antibody directed against Wnt can be made using standard techniques. For example, human monoclonal antibodies can be generated in transgenic mice or in immune deficient mice engrafted with antibody-producing human cells. Methods of generating such mice are describe, for example, in Wood et al. PCT publication WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg et al. PCT

publication WO 92/03918; Kay et al. PCT publication WO 92/03917; Kay et al. PCT publication WO 93/12227; Kay et al. PCT publication 94/25585; Rajewsky et al. Pct publication WO 94/04667; Ditullio et al. PCT publication WO 95/17085; Lonberg, N. et al. (1994) *Nature* 368:856-859; Green, L.L. et al. (1994) *Nature Genet.* 7:13-21; Morrison, S.L. et al. (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman et al. (1993) *Year Immunol* 7:33-40; Choi et al. (1993) *Nature Genet.* 4:117-123; Tuaillon et al. (1993) *PNAS* 90:3720-3724; Bruggeman et al. (1991) *Eur J Immunol* 21:1323-1326); Duchosal et al. PCT publication WO 93/05796; U.S. Patent Number 5,411,749; McCune et al. (1988) *Science* 241:1632-1639), Kamel-Reid et al. (1988) *Science* 242:1706; Spanopoulou (1994) *Genes & Development* 8:1030-1042; Shinkai et al. (1992) *Cell* 68:855-868). A human antibody-transgenic mouse or an immune deficient mouse engrafted with human antibody-producing cells or tissue can be immunized with Wnt or an antigenic Wnt peptide and splenocytes from these immunized mice can then be used to create hybridomas. Methods of hybridoma production are well known.

Human monoclonal antibodies against Wnt can also be prepared by constructing a combinatorial immunoglobulin library, such as a Fab phage display library or a scFv phage display library, using immunoglobulin light chain and heavy chain cDNAs prepared from mRNA derived from lymphocytes of a subject. *See, e.g.*, McCafferty et al. PCT publication WO 92/01047; Marks et al. (1991) *J. Mol. Biol.* 222:581-597; and Griffths et al. (1993) *EMBO J* 12:725-734. In addition, a combinatorial library of antibody variable regions can be generated by mutating a known human antibody. For example, a variable region of a human antibody known to bind Wnt, can be mutated, by for example using randomly altered mutagenized oligonucleotides, to generate a library of mutated variable regions which can then be screened to bind to Wnt. Methods of inducing random mutagenesis within the CDR regions of immunoglobin heavy and/or light chains, methods of crossing randomized heavy and light chains to form pairings and screening methods can be found in, for example, Barbas et al. PCT publication WO 96/07754; Barbas et al. (1992) *Proc. Nat'l Acad. Sci. USA* 89:4457-4461.

The immunoglobulin library can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Examples of methods and reagents particularly amenable for use in generating antibody display library

can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. PCT publication WO 92/18619; Dower et al. PCT publication WO 91/17271; Winter et al. PCT publication WO 92/20791; Markland et al. PCT publication WO 92/15679; Breitling et al. PCT publication WO 93/01288; McCafferty et al. PCT publication WO 92/01047; Garrard et al. PCT publication WO 92/09690; Ladner et al. PCT publication WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *supra*; Hawkins et al. (1992) *J Mol Biol* 226:889-896; Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982. Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened to identify and isolate packages that express an antibody that binds Wnt. In a preferred embodiment, the primary screening of the library involves panning with an immobilized Wnt and display packages expressing antibodies that bind immobilized Wnt are selected.

Antisense Wnt Nucleic Acid Sequences

Nucleic acid molecules which are antisense to a nucleotide encoding Wnt can be used as an agent which inhibits Wnt expression. An "antisense" nucleic acid includes a nucleotide sequence which is complementary to a "sense" nucleic acid encoding Wnt, e.g., complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. Accordingly, an antisense nucleic acid can form hydrogen bonds with a sense nucleic acid. The antisense nucleic acid can be complementary to an entire Wnt coding strand, or to only a portion thereof. For example, an antisense nucleic acid molecule which antisense to the "coding region" of the coding strand of a nucleotide sequence encoding Wnt can be used.

The coding strand sequences encoding Wnt are known. For example, at least 7 Wnt genes have been identified in human (Wnt-1, 2, 3, 4, 5a, 7a and 7b). See, e.g., Vant Veer et al. (1984) Mol. Cell. Biol. 4:2532-2534, Gavin et al. (1992) *Gene Dev.* 4:2319-2332; Lee et al. (1995) *Proc. Natl Acad. Sci USA* 92:2268-2272; Christiansen et al. (1995) Mech. Dev. 51:341-350, Wainwright et al. (1988) EMBO J. 7:1743-1748; and, PCT Publication WO

95/17416. Given the coding strand sequences encoding Wnt, antisense nucleic acids can be designed according to the rules of Watson and Crick base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of Wnt mRNA, but more preferably is an oligonucleotide which is antisense to only a portion of the coding or noncoding region of Wnt mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of Wnt mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (e.g., an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, e.g., phosphorothioate derivatives and acridine substituted nucleotides can be used. Examples of modified nucleotides which can be used to generate the antisense nucleic acid include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e., RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest.

Gene Therapy

The gene constructs of the invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a Wnt polypeptide. The invention features expression vectors for *in vivo* transfection and expression of a Wnt polypeptide in particular cell types so as to reconstitute the function of, or alternatively, antagonize the function of a Wnt polypeptide in a cell in which that polypeptide is misexpressed. Expression constructs of Wnt polypeptides, may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the Wnt gene to cells *in vivo*. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out *in vivo*.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding a Wnt polypeptide. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A replication defective retrovirus can be packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro*

or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψCrip, ψ Cre, ψ2 and ψAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited *supra*). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential

problems that can occur as a result of insertional mutagenesis *in situ* where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267).

Yet another viral vector system useful for delivery of the subject gene is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. (1992) *Curr. Topics in Micro. and Immunol.* 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a Wnt polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject Wnt gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding a Wnt polypeptide can be entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally)

which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al. (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075).

In clinical settings, the gene delivery systems for the therapeutic Wnt gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced in tact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In other preferred embodiments, ex vivo gene therapy approaches can be used.

Cell Therapy

Wnt can also be increased in a subject by introducing into a cell, e.g., a fibroblast, keratinocyte, epithelial cell, e.g., a hair follicle cell, e.g., a DP cell, a nucleotide sequence that modulates the production of Wnt, e.g., a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof, a promoter sequence, e.g., a promoter sequence from a Wnt gene or from another gene; an enhancer sequence, e.g., 5' untranslated region (UTR), e.g., a 5' UTR from a Wnt gene or from another gene, a 3' UTR, e.g., a 3' UTR from a Wnt gene or from another gene; a polyadenylation site; an insulator sequence; or another

sequence that modulates the expression of Wnt. The cell can then be introduced into the subject.

Primary and secondary cells to be genetically engineered can be obtained from a variety of tissues and include cell types which can be maintained propagated in culture. For example, primary and secondary cells include fibroblasts, keratinocytes, epithelial cells (e.g., DP cells), endothelial cells, glial cells, neural cells, formed elements of the blood (e.g., lymphocytes, bone marrow cells), muscle cells (myoblasts) and precursors of these somatic cell types. Primary cells are preferably obtained from the individual to whom the genetically engineered primary or secondary cells are administered. However, primary cells may be obtained for a donor (other than the recipient) of the same species or another species (e.g., mouse, rat, rabbit, cat, dog, pig, cow, bird, sheep, goat, horse).

The term "primary cell" includes cells present in a suspension of cells isolated from a vertebrate tissue source (prior to their being plated i.e., attached to a tissue culture substrate such as a dish or flask), cells present in an explant derived from tissue, both of the previous types of cells plated for the first time, and cell suspensions derived from these plated cells. The term "secondary cell" or "cell strain" refers to cells at all subsequent steps in culturing. That is, the first time a plated primary cell is removed from the culture substrate and replated (passaged), it is referred to herein as a secondary cell, as are all cells in subsequent passages. Secondary cells are cell strains which consist of secondary cells which have been passaged one or more times. A cell strain consists of secondary cells that: 1) have been passaged one or more times; 2) exhibit a finite number of mean population doublings in culture; 3) exhibit the properties of contact-inhibited, anchorage dependent growth (anchorage-dependence does not apply to cells that are propagated in suspension culture); and 4) are not immortalized. A "clonal cell strain" is defined as a cell strain that is derived from a single founder cell. A "heterogenous cell strain" is defined as a cell strain that is derived from two or more founder cells.

Primary or secondary cells of vertebrate, particularly mammalian, origin can be transfected with an exogenous nucleic acid sequence which includes a nucleic acid sequence encoding a signal peptide, and/or a heterologous nucleic acid sequence, e.g., encoding Wnt, and produce the encoded product stably and reproducibly *in vitro* and *in vivo*, over extended periods of time. A heterologous amino acid can also be a regulatory sequence, e.g., a

promoter, which causes expression, e.g., inducible expression or upregulation, of an endogenous Wnt sequence. An exogenous nucleic acid sequence can be introduced into a primary or secondary cell by homologous recombination as described, for example, in U.S. Patent No.: 5,641,670, the contents of which are incorporated herein by reference.

The transfected primary or secondary cells may also include DNA encoding a selectable marker which confers a selectable phenotype upon them, facilitating their identification and isolation. Methods for producing transfected primary and secondary cells which stably express exogenous synthetic DNA, clonal cell strains and heterogeneous cell strains of such transfected cells, methods of producing the clonal heterogeneous cell strains, and methods of treating or preventing an abnormal or undesirable condition through the use of populations of transfected primary or secondary cells are part of the present invention.

Transfection of Primary or Secondary Cells of Clonal or Heterogeneous Cell Strains

Vertebrate tissue can be obtained by standard methods such a punch biopsy or other surgical methods of obtaining a tissue source of the primary cell type of interest. For example, punch biopsy or removal of a hair follicle is used to obtain a source of fibroblasts, keratinocytes, or endothelial cells, e.g., hair follicle cells or DP cells. A mixture of primary cells is obtained from the tissue, using known methods, such as enzymatic digestion or explanting. If enzymatic digestion is used, enzymes such as collagenase, hyaluronidase, dispase, pronase, trypsin, elastase and chymotrypsin can be used.

The resulting primary cell mixture can be transfected directly or it can be cultured first, removed from the culture plate and resuspended before transfection is carried out. Primary cells or secondary cells are combined with exogenous nucleic acid sequence to, e.g., stably integrate into their genomes, and treated in order to accomplish transfection. The exogenous nucleic acid sequence can optionally include DNA encoding a selectable marker. The exogenous nucleic acid sequence and selectable marker-encoding DNA can either be on separate constructs or on a single construct. An appropriate quantity of DNA is used to ensure that at least one stably transfected cell containing and appropriately expressing exogenous DNA is produced. In general, approximately 0.1 to 500 μg of DNA is used.

As used herein, the term "transfection" includes a variety of techniques for introducing an exogenous nucleic acid into a cell including calcium phosphate or calcium

chloride precipitation, microinjection, DEAE-dextrin-mediated transfection, lipofection or electrophoration.

Electroporation is carried out at approximate voltage and capacitance (and corresponding time constant) to result in entry of the DNA construct(s) into the primary or secondary cells. Electroporation can be carried out over a wide range of voltages (e.g., 50 to 2000 volts) and corresponding capacitance. Total DNA of approximately 0.1 to 500μg is generally used.

Methods such as calcium phosphate precipitation, modified calcium phosphate precipitation an polybrene precipitation, liposome fusion and receptor-mediated gene delivery can also be used to transect cells. Primary or secondary cells can also be transfected using microinjection. A stably, transfected cell can then be isolated and cultured and sub cultivated, under culturing conditions and for sufficient time to propagate stably transfected secondary cells an produce a clonal cell strain of transfected secondary cells. Alternatively, more than one transfected cell is cultured and sub cultured, resulting in production of a heterogeneous cell strain.

Transfected primary or secondary cells undergo sufficient number doubling to produce either a clonal cell strain or a heterogeneous cell strain of sufficient size to provide the therapeutic protein to an individual in effective amounts. In general, for example, $0.1cm^2$ of skin is biopsies and assumed to contain 1,000,000 cells; one cell is used to produce a clonal cell strain and undergoes approximately 27 doublings to produce 100 million transfected secondary cells. If a heterogeneous cell strain is to be produced from an original transfected population of approximately 1000,000 cells, only 10 doublings are needed to produce 100 million transfected cells.

The number of required cells in a transfected clonal heterogeneous cell strain is variable and depends on a variety of factors, including but not limited to, the use of the transfected cells, the functional level of the exogenous DNA in the transfected cells, the site of implantation of the transfected cells (for example, the number of cells that can be used is limited by the anatomical site of implantation), and the age, surface area, and clinical condition of the patient. The put these factors in perspective, to deliver therapeutic levels of human growth hormone in an otherwise healthy 10 kg patient with isolated growth hormone

deficiency, approximately one to five hundred million transfected fibroblast would be necessary (the volume of these cells is about that of the very tip of the patient's thumb).

Implantation of Clonal Cell Strain or Heterogeneous Cell Strain of Transfected Secondary Cells

The transfected cells, e.g., cells produced as described herein, can be introduced into an individual to whom the product is to be delivered. The clonal cell strain or heterogeneous cell strain is then introduced into an individual. Various routed of administration and various sites (e.g., renal sub capsular, subcutaneous, central nervous system (including intrathecal), intravascular, intrahepatic, intrasplanchnic, intraperitoneal (including intraomental), intramuscularly implantation) can be used. One implanted in individual, the transfected cells produce the product encoded by the heterologous DNA or are affected by the heterologous DNA itself. For example, an individual who suffers from a condition related to unwanted angiogenesis is a candidate for implantation of Wnt producing cells.

The individual can have a small skin biopsy performed; this is a simple procedure which can be performed on an outpatient basis. The piece of skin is taken, for example, from under the arm and can require about one minute to remove. The sample is processed, resulting in isolation of the patient's cell (e.g., fibroblasts) and genetically engineered to produce Wnt or another protein or molecule that induces the production of Wnt. Based on the age, weight, and clinical condition of the patient, the required number of cells are grown in large-scale culture. The entire process should require 4-6 weeks and, at the end of that time, the appropriate number of genetically engineered cells are introduced into the individual, once again as an outpatient (e.g., by injecting them back under the patient's skin, e.g., on the scalp or face). The patient is now capable of producing Wnt which can ameliorate symptoms of hair loss.

For some, this will be a one-time treatment and, for others, multiple cell therapy treatments will be required.

As this example suggests, the cells used will generally be patient-specific genetically engineered cells. It is possible, however, to obtain cells from another individual of the same species or from a different species. Use of such cells might require administration of an

immunosuppressant, alteration of histocompatibility antigens, or use of a barrier device to prevent rejection of the implanted cells.

Transfected primary or secondary cells can be administered alone or in conjunction with a barrier or agent for inhibiting immune response against the cell in a recipient subject. For example, an immunosuppressive agent can be administered to a subject to inhibit or interfere with normal response in the subject. Preferably, the immunosuppressive agent is an immunosuppressive drug which inhibits T cell/or B cell activity in a subject. Examples of such immunosuppressive drugs commercially available (e.g., cyclosporin A is commercially available from Sandoz Corp. East Hanover, NJ).

An immunosuppressive agent, e.g., drug, can be administered to a subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of the cells). Dosage ranges for immunosuppressive drugs are known in the art. *See, e.g.*, Freed et al. (1992) *N. Engl. J. Med.* 327:1549; Spencer et al. (1992) *N. Engl. J. Med.* 327:1541' Widner et al. (1992) *n. Engl. J. Med.* 327:1556). Dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual.

Another agent with can be used to inhibit T cell activity in a subject is an antibody, or fragment of derivative thereof. Antibodies capable of depleting or sequestering T cells *in vivo* are known in the art. Polyclonal antisera can be used, for example, anti-lymphocyte serum. Alternatively, one or more monoclonal antibodies can be used. Preferred T cell depleting antibodies include monoclonal antibodies which bind to CD2, CD3, CD4, CD8, CD40, CD40, ligand on the cell surface. Such antibodies are known in the art and are commercially available, for example, from American Type Culture Collection. A preferred antibody for binding CD3 on human T cells is OKT3 (ATCC CRL 8001).

An antibody which depletes, sequesters or inhibits T cells within a recipient subject can be administered in a dose for an appropriate time to inhibit rejection of cells upon transplantation. Antibodies are preferably administered intravenously in a pharmaceutically acceptable carrier of diluent (e.g., saline solution).

An advantage of the use of transfected or secondary cells is that by controlling the number of cells introduced into an individual, one can control the amount of the protein delivered to the body. In addition, in some cases, it is possible to remove the transfected cells of there is no longer a need for the product. A further advantage of treatment by use of

transfected primary or secondary cells of the present invention is that production of the therapeutic product can be regulated, such as through the an administration of zinc, steroids or an agent which affects transcription of a protein, product or nucleic acid product or affects the stability of a nucleic acid product.

Administration

An agent which modulates the level of expression of a Wnt protein can be administered to a subject by standard methods. For example, the agent can be administered by any of a number of different routes including intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), and transmucosal. In one embodiment, the Wnt modulating agent can be administered topically.

The agent which modulates Wnt protein levels, e.g., nucleic acid molecules, Wnt polypeptides, fragments or analogs, Wnt modulators, and anti-Wnt antibodies (also referred to herein as "active compounds") can be incorporated into pharmaceutical compositions suitable for administration to a subject, e.g., a human. Such compositions typically include the nucleic acid molecule, polypeptide, modulator, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances are known. Except insofar as any conventional media or agent is incompatible with the active compound, such media can be used in the compositions of the invention. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition can be formulated to be compatible with its intended route of administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with

acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a Wnt polypeptide or anti-Wnt antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. Such transdermal formulations can by applied to the skin to promote or inhibit hair growth.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be

prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The nucleic acid molecules described herein can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al., *PNAS* 91:3054-3057, 1994). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The agent which modulates the level of Wnt protein can be administered by locally administration, e.g., topical administration. The agent can be applied once or it can be administered continuously, e.g., the agent is administered with sufficient frequency such that the affect on the Wnt protein level is maintained for a selected period, e.g., 5, 10, 20, 30, 50, 90, 180, 365 days or more. The administration of an agent which modulates, e.g., increases or inhibits, the level of a Wnt protein, e.g., a Wnt polypeptide or an anti-Wnt antibody, can also be repeated.

Other Embodiments

It is understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

All patents and references cited herein are incorporated in their entirety by reference. Other embodiments are within the following claims.

What is claimed:

1. A method of promoting hair growth in a subject, comprising inducing or mimicking an effect of Wnt-promoted signal transduction in a subject, thereby promoting hair growth.

2. The method of claim 1, wherein Wnt-promoted signal transduction is induced or mimicked in a dermal papilla cell of the subject.

3. The method of claim 1, wherein an effect of Wnt promoted signal transduction is induced by administering to the subject an agent which increases the level of Wnt protein production or activity.

4. The method of claim 3, wherein the agent is a Wnt polypeptide or a functional fragment or analog thereof.

5. The method of claim 3, wherein the agent is a nucleotide sequence encoding a Wnt polypeptide or functional fragment or analog thereof.

6. The method of claim 5, wherein the Wnt polypeptide is Wnt3, Wnt 4, or Wnt 7.

7. The method of claim 1, wherein an effect of Wnt promoted signal transduction is mimicked by administering to the subject an agent that inhibits β-catenin phosphorylation or inhibits GSK3β kinase.

8. The method of claim 7, wherein the agent is lithium chloride.

9. The method of claim 1, wherein an effect of Wnt promoted signal transduction is mimicked by administering to the subject an agent that increases cytoplasmic accumulation of β-catenin.

10. The method of claim 1, wherein an effect of Wnt promoted signal transduction is mimicked by an agent that interacts with a Frizzled receptor.

11. The method of claim 10, wherein the agent is an antibody.

12. A method of inhibiting hair growth in a subject, comprising inhibiting the level of Wnt protein or inhibiting an effect of Wnt-promoted signal transduction, in a subject.

13. The method of claim 12, wherein the level of Wnt protein or Wnt-promoted signal transduction is inhibited in a dermal papilla cell.

14. The method of claim 12, wherein an effect of Wnt-promoted signal transduction is inhibited by administering to the subject an agent which decreases the level of Wnt protein production or activity.

15. The method of claim 12, wherein the Wnt protein is a Wnt3, Wnt 4, or Wnt 7.

16. The method of claim 12, wherein an effect of Wnt-promoted signal transduction is inhibited by an agent that increases β-catenin phosphorylation or induces GSK3β kinase activity.

17. The method of claim 12, wherein an effect of Wnt promoted signal transduction is inhibited by administering an agent that decreases cytoplasmic accumulation of β-catenin.

18. A method of evaluating whether a subject is at risk for hair loss, comprising detecting the presence or absence of a genetic lesion in a Wnt gene or misexpression of a Wnt gene, thereby evaluating whether a subject is at risk for hair loss.

19. The method of claim 18, wherein the Wnt gene Wnt3, Wnt 4, or Wnt 7.

20. The method of claim 18, wherein the genetic lesion or misexpression is detected in a hair follicle of the subject.

21. The method of claim 18, wherein underexpression of Wnt is indicative of a risk of hair loss.

22. A method of identifying a compound capable of promoting hair growth, comprising:

contacting a cell capable of expressing a Wnt polypeptide with a test compound; and

determining the level of Wnt polypeptide or nucleic acid expression in the cell, wherein a compound capable of increasing Wnt polypeptide or nucleic acid expression is indicative of a compound capable of promoting hair growth,

thereby identifying a compound capable of promoting hair growth.

23. The method of claim 22, wherein the Wnt polypeptide is Wnt 3, Wnt 4, or Wnt 7.

24. The method of claim 22, wherein the test compound is a Wnt fragment or analog.

25. The method of claim 22, wherein the cell is a hair follicle cell.

26. A method of identifying a compound capable of inhibiting hair growth, comprising:

contacting a cell capable of expressing a Wnt polypeptide with a test compound; and

determining the level of Wnt polypeptide or nucleic acid expression in the cell, wherein a compound capable of decreasing Wnt polypeptide or nucleic acid expression is indicative of a compound capable of inhibiting hair growth,

thereby identifying a compound capable of inhibiting hair growth.

27. The method of claim 26, wherein the Wnt polypeptide is Wnt 3, Wnt 4, or Wnt 7.

28. The method of claim 26, wherein the test compound is a Wnt antagonist.

29. The method of claim 26, wherein the cell is a hair follicle cell.

30. A method of culturing a dermal papilla (DP) cell, comprising culturing the DP cell in the presence of an increased level of Wnt or an agent which mimics an effect of Wnt-promoted signal transduction.

31. The method of claim 31, wherein the agent is lithium chloride.

32. The method of claim 30, wherein a Wnt polypeptide or a functional fragment or analog thereof is added to the culture.

33. The method of claim 30, wherein the DP cell is cultured in the presence of a cell which expresses a Wnt polypeptide or a functional fragment or analog thereof.

34. A culture media for dermal papilla (DP) cells, comprising: a Wnt polypeptide or a functional fragment or analog thereof, or an agent which mimics an effect of Wnt promoted signal transduction.

35. The culture media of claim 34, wherein the agent is lithium chloride.

36. A method of promoting or maintaining anagen phase gene expression in a dermal papilla (DP) cell, comprising: increasing the level of Wnt protein or mimicking an effect of Wnt promoted signal transduction in the DP cell.

37. The method of claim 36, wherein the Wnt protein is Wnt3, Wnt 4, or Wnt 7.

38. The method of claim 37, wherein mimicking an effect of Wnt promoted signal transduction comprises inhibiting β-catenin phosphorylation in the DP cell.

39. The method of claim 38, wherein mimicking an effect of Wnt promoted signal transduction in the DP cell comprises contacting the cell with lithium chloride.

40. A method of providing and maintaining a dermal papilla (DP) cell graft comprising:

providing a DP cell from a subject; and

culturing the DP cell under conditions that induce or mimic an effect of Wnt-promoted signal transduction, thereby providing and maintaining a dermal papilla (DP) cell graft.

41. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of Wnt or a fragment or analog thereof.

42. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of lithium chloride.

43. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of β-catenin and/or LEF-1.

44. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of an agent which inhibits β-catenin phosphorylation.

45. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of an agent which inhibits GSK3β kinase.

46. The method of claim 40, wherein the dermal papilla cell is cultured in the presence of an agent which promotes accumulation of β-catenin.

47. The method of claim 40, further comprising the step of returning the DP cell to the same or a different subject.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/10164

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :435/325, 375, 455; 514/ 2, 44, 880

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/325, 375, 455; 514/ 2, 44, 880

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN (Bioscience), EAST (all databases), search terms: Wnt, hair growth, alopecia, follicle, derma papilla, beta-catenin, GSKs-beta kinase, lithium chloride, frizzled, anagen.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P --- A, P	US 6,159,950 A (CRYSTAL et al.) 12 December 2000, entire document, especially Col. 3, lines 49-51, Col. 4, lines 18-21, Col. 5, lines 25-28.	1-3, 12-14, 30, 36 --- 1-47
A	CHUONG et al. Early Events During Avian Skin Appendage Regeneration: Dependene on Epithelial-Mesenchymal Interaction and Order of Molecular Reappearance. J. Invest. Dermatol. 1996, Vol. 107, pp. 639-646.	1-47
X, P --- A, P	CHARPENTIER et al. Plakoglobin Suppresses Epithelial Proliferation and Hair Growth In Vivo. J. Cell Biol. April 17, 2000, Vol. 149, No. 2, pages 503-519, especially p. 503, last two lines- p. 504, line 4, and p. 513, paragraph 3.	12 --- 1-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 14 JUNE 2001	Date of mailing of the international search report 27 JUL 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer HOLLY SCHNIZER Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/10164

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A01N 37/18; A61K 38/00, 48/00; C12N 5/02, 15/ 63, 15/64, 15/85, 15/87, 15/90

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/10164

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	MILLAR et al. WNT Signaling in the Control of Hair Growth and Structure. Dev. Biol. 1999, Vol. 207, pages 133-149, especially p. 133, abstract, lines 3-5 and 9-10, p. 135, Col. 1, last paragraph.	12-15 --- 1-47
X, P --- A, P	KISHIMOTO et al. Wnt Signaling maintains the hair-inducing activity of the dermal papilla. Genes and Development, 2000, Vol. 14, pages 1181-1185, especially abstract, p. 1183, p. 1184, Col. 1.	1-7, 9, 10, 30, 32, 34, 36-38 --- 1-47

Form PCT/ISA/210 (continuation of second sheet) (July 1998)*